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Functional polyesters produced by pseudomonads/

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FUNCTIONAL POLYESTERS PRODUCED BY PSEUDOMONADS

A Dissertation Presented

by

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Polymer Science and Engineering Department

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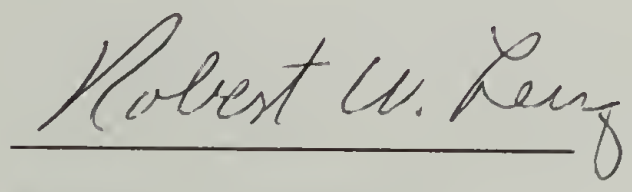

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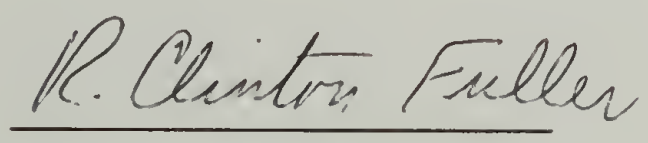

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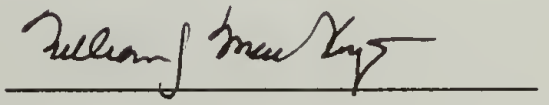

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ABSTRACT

FUNCTIONAL POLYESTERS PRODUCED BY PSEUDOMONADS

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The ability of two different pseudomonads, *Pseudomonas oleovorans* and *Pseudomonas putida* to metabolize and produce polymers from functional carbon substrates, in particular phenyl-containing substrates was investigated. A comparison of the two bacteria revealed *P. putida* to be the superior microorganism in that it produced higher cell yields and polymer yields regardless of the substrate on which it was grown.

The bacterial polyester produced when *P. oleovorans* was grown on a mixture of 5-phenylvaleric acid and nonanoic acid, included both a homopolymer , poly-3-hydroxyphenylvalerate (PHPV) and a copolymer, poly-3-hydroxynonanoate (PHN). The intracellular location of each of these polymers was determined by selective staining of the inclusion body granules with ruthenium tetraoxide. Examination of the stained granules by transmission electron microscopy showed that both types of polyesters occurred sequentially in the same granule. Poly-3-hydroxynonanoate was synthesized first, and was present in the center of the granule, while poly-3-hydroxyphenylvalerate accumulated afterwards around the PHN inclusion body. The enzymes associated with the inclusion bodies were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In all cases, two polymerase enzymes of molecular weights of 59 and 55 KD were present, which suggests that the same polymerase enzymes may have been responsible for the production of both PHN and PHPV. In addition, attempts were made to produce a random copolymer containing both alkyl and phenylalkyl repeat unit by varying the growth conditions, but a mixture of two polymers was produced instead.

PHPV is a homopolymer and 100% isotactic. However, it does not exhibit crystallinity. Various different annealing experiments carried out on PHPV did not result in the induction of crystallinity. However, a crystalline phenyl-containing polymer, poly-3-hydroxy-5-(4'-tolyl)valerate was produced when either *P. oleovorans* or *P. putida* was grown on 5-(4'-tolyl)valeric acid. To the best of our knowledge, this is the first report of a crystalline phenyl-containing microbially produced polyester.

Intracellular degradation studies were carried out on *P. oleovorans* cells which contained either PHN, PHPV or a blend of PHN/PHPV. Fairly rapid intracellular degradation occurred when PHN was the sole storage polymer. The intracellular degradation rate of PHPV was much slower. However, when both polymers were present as intracellular storage polymer the rate of degradation of PHPV was greater than when PHPV was the sole intracellular storage polymer. It is possible that both polymers are degraded by the same intracellular depolymerase, which is more efficient in the presence of PHN.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Introduction

Poly-3-hydroxyalkanoates (PHA's) are a class of polyesters produeed by a number of micro-organisms as a storage material when they experience metabolie stress, such as a limitation of nitrogen, oxygen or other essential nutrients, in the presence of an excess of carbon source [1,2,3]. Bacteria will divide only when all essential nutrients are present. When one of the essential nutrients is absent, the culture will stop growing and enter the stationary phase. At this point, if there is a excess of one essential nutrient the bacteria will store it in a highly reduced form which will be consumed later when growth is once again possible. Some of the nutrients stored by bacteria inelude glycogen, polyphosphates, polysulfurs and poly-3-hydroxyalkanoates. PHA's are an excellent storage material as they are highly reduced, and exert very little osmotic pressure. These polyesters have the basic structure illustrated in Figure 1.1 ; the -R group is dependent upon the carbon substrate.

Each repeat unit of a PHA has a chiral center at the b position, (from the carbonyl group) and all repeat units are in the [R] configuration. This point is essential to the inherent degradability of these biopolymers, and is thought to be one of the main reasons for the lack of degradability of chemically synthesized polyesters which have similar structures but lack the stereoregularity of natural PHA's [4].

The micro-organisms which produce poly-3-hydroxyalkanoate polymers can be divided into two classes. Those which store polyester with short side-chains where R=1-2 and those which produce PHA's with longer sidechains where R=3-9. *Alcaligenes euthrophus, Rhodospirillum rubrum* and *Rhodobacter sphaeroides* are some of the more well known bacteria which metabolize carboxylic acids of seven carbons or less to produee PHA's which have either 1 or 2 carbons in the sidechain. Lemoigne was the first person to

[5, 6]. In 1959 and 1960 Baptist obtained the first patents on PHB in which he stated that this polymer could be used as a biodegradable suture [7,8]. PHB is a highly crystalline polymer (more than 50% crystalline) with a glass transition temperature (Tg) of 5^0C and a melting temperature (Tm) of 180^0C which is produced when *Alcaligenes eutrophus* is fed glucose as a carbon source. The structure of PHB is shown in Figure 1.2.

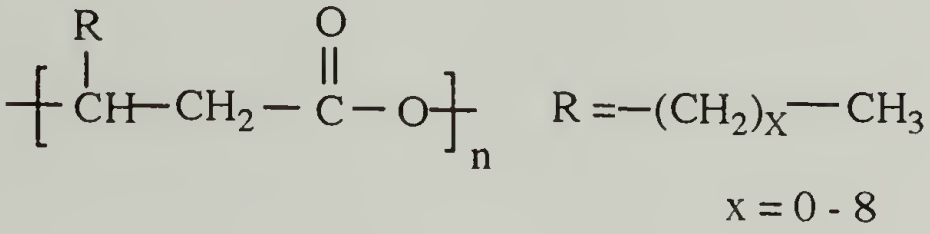
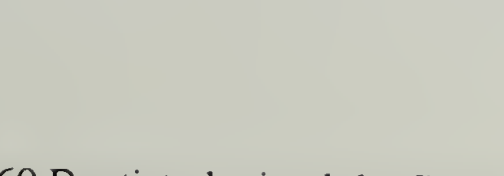


Figure 1.1 The general chemical structure of poly-3-hydroxyalkanoates



Poly-3-hydroxybutyrate
PHB

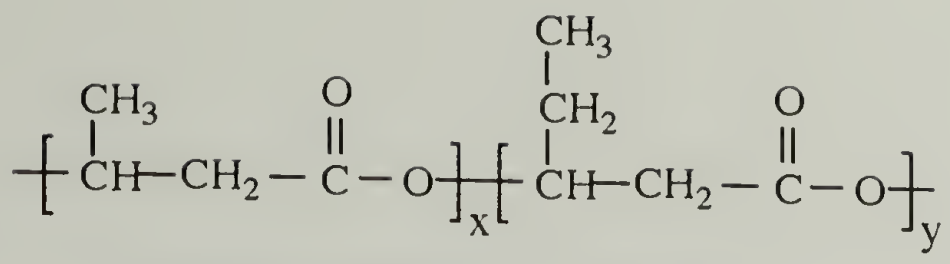
Figure 1.2 The chemical structure of poly-3-hydroxybutyrate (PHB)

The next type of bacterial polyesters discovered was isolated from sludge by Wallen in 1974 [9]. It was composed of mainly 3-hydroxybutyrate, and 3-hydroxyvalerate, but also contained 3-hydroxyhexanoate, 3-hydroxyheptanoate and 3-hydroxyoctanoate as minor components. The chemical structure of the PHBV copolymer can be seen in Figure 1.3.

Since then, extensive research has been carried out on these short chain PHA's. Zeneca is currently commercially producing PHBV (under the trade name 'Biopol') in Billington, England by feeding *Alcaligenes eutrophus* a mixture of glucose and propionic acid [10,11]. The copolymer is commercially available in the range of 0-30% HV repeat units. Doi reported the incorporation of up to 85% 3-hydroxyvalerate repeat units into the PHBV copolymer by feeding the bacterium pentanoic acid as a sole carbon source [12, 13]. While *A. eutrophus* is not a highly versatile bacterium, some novel polymers have been produced. For example, a copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate P(3HB-co-4HB) (Figure 1.4) was produced when the bacterium was grown on butyric acid and 4-hydroxybutyric acid. Up to 33% 4HB repeat units were obtained when the bacterium was grown on 4-hydroxybutyric acid as a sole carbon source [14-16]. Also a terpolymer of 3-hydroxybutyrate, 3-hydroxyvalerate and 5-hydroxyvalerate (Figure 1.5) was produced when *Alcaligenes eutrophus* was grown on an equimolar mixture of 5-chloropentanoic acid and pentanoic acid [17]. All of these copolymers are statistically random [14].

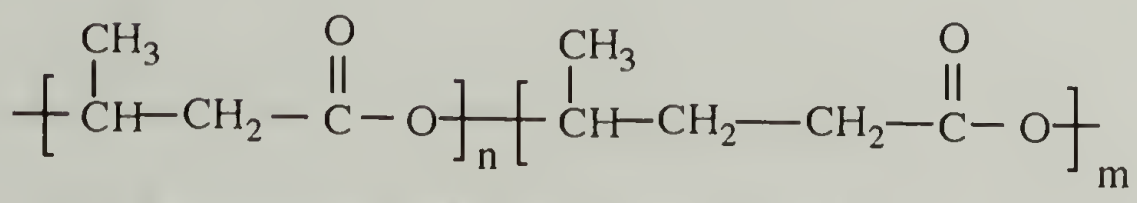
There have been a number of review articles published on the subject of PHB and PHBV, in which the mechanism for polymer production, physical characterization and the degradation have been discussed [18-20].

De Smet and coworkers discovered that the inclusion bodies which were isolated from *Pseudomonas. oleovorans* grown on octane were poly-3-hydroxyoctanoate (PHO) [21]. PHO is a random copolymer with 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate as the repeat units (Figure 1.6), Since then it has been determined that *P. oleovorans* can grow on alkanes, alcohols and alkanoic acids, and on carbon source of chain length between 1 and 16 and produce PHA's where the R group can vary in length from 3-9 carbons. The results obtained when *P. oleovorans* was grown on substrates varying in length from C4 to C16 are summarized in Table 1.1 [22].



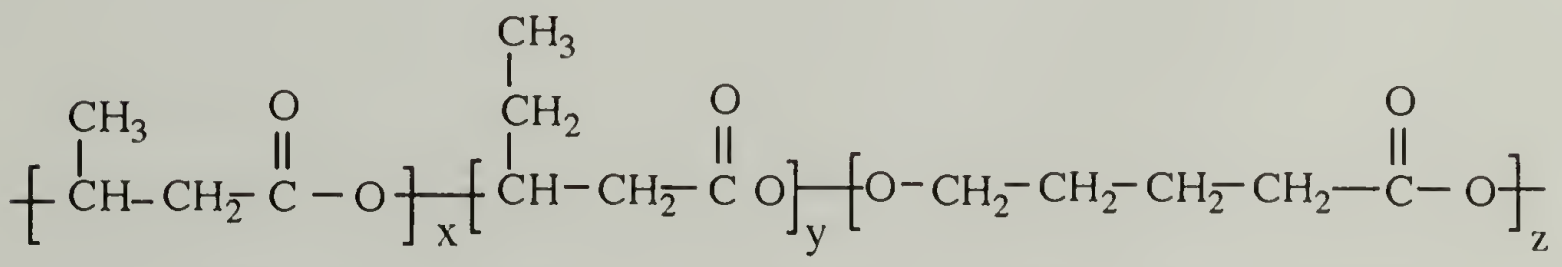
Poly-3-hydroxybutyrate-co-valerate
PHBV

Figure 1.3 The chemical structure of the copolymer poly-3-hydroxybutyrate-co-valerate (PHBV)



Poly-3-hydroxybutyrate-co-4-hydroxyvalerate
P(3HB-co-4HB)

Figure 1.4 The chemical structure of the copolymer poly-3-hydroxybutyrate-co-4-hydroxybutyrate (P(3HB-co-4HB) obtained when *Alcaligenes eutrophus* was grown on a mixture of butyric acid and 4-hydroxybutyric acid.



Terpolymer of 3-hydroxybutyrate, 3-hydroxyvalerate, 5-hydroxyvalerate

Figure 1.5 The chemical structure of the terpolymer obtained when *A. eutrophus* was grown on a mixture of 5-chloropentanoic acid and pentanoic acid.

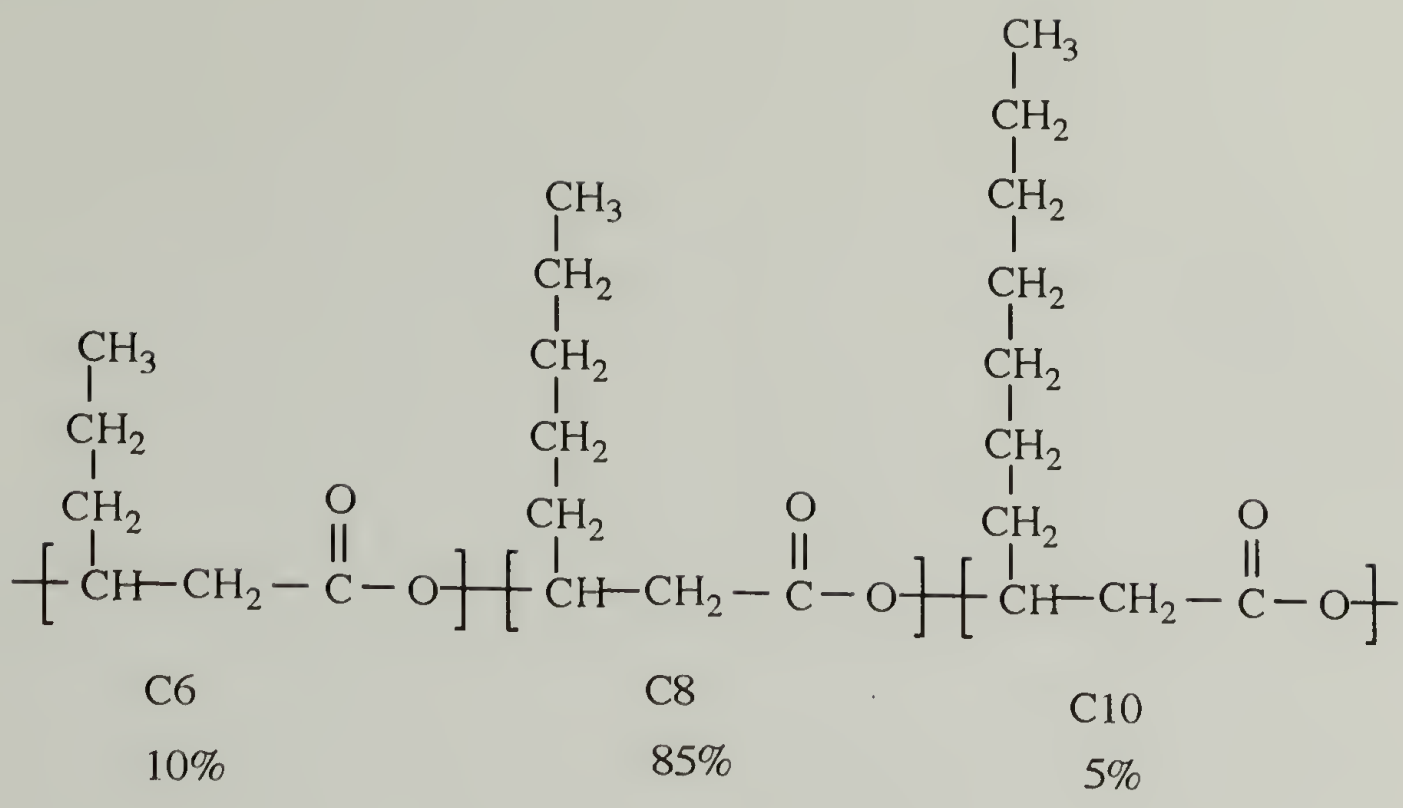


Figure 1.6 The chemical structure and repeating unit composition of poly-3-hydroxyoctanoate (PHO), the polymer obtained when *Pseudomonas oleovorans* was grown on octanoic acid.

Table 1.1 Accumulation and composition of PHA's obtained from *P. oleovorans* grown on various substrates [22].

Substrate	PHA content Weight %	PHA Composition (mole % 3HA units)						
		C6	C7	C8	C9	C10	C11	C12
3-hydroxybutyrate	1.2	---	---	22	---	57	---	21
butyrate	0.6	---	---	---	---	33	---	67
Valerate	0.7	---	---	---	---	35	---	65
Hexanoate	3.3	95	---	5	---	---	---	---
Heptanoate	2.3	---	100	---	---	---	---	---
Octanoate	8.7	8	---	91	---	1	---	---
Nonanoate	9.1	---	35	---	65	---	---	---
Decanoate	12.5	8	---	75	---	17	---	---
Undecanoate	9.8	---	28	---	59	---	13	---
Dodecanoate	6.6	6	---	57	---	32	---	5
Tridecanoate	5.4	---	32	---	48	5	14	---
Tetradecanaote	10.6	7	---	58	---	30	---	4
Pentadecanoate	5.3	---	32	---	47	8	13	---
Hexadecanoate	3.4	8	---	50	---	30	---	12
Heptadecanoate	no growth							
Octadecanoate	no growth							

As is evident from Table 1.1, the major constituent when the bacteria was grown on octane, octanoic acid or octanol was 3-hydroxyoctanoate, and 3-hydroxynonanoate was the major constituent when the bacteria was grown on nonane, nonanoic acid, or nonanol. Also when the bacteria were grown on a carbon source with more than nine carbon atoms , 3-hydroxyoctanoate was the major constituent if the carbon source contains an even number of carbons and 3-hydroxynonanoate is the major constituent if the substrate used contains an odd number of carbons [23-26].

P. oleovorans has proven to be quite a versatile bacterium, and many functional substrates, not normally found in nature, can be metabolized to produce polyesters with these functional groups in the sidechain. The incorporation of functional groups within the polymer chain opens up the possibilities of producing biopolymers with various properties, and also presents the opportunity to chemically modify these biopolymers.

The various functional groups fed to *P. oleovorans* were divided into three groups by Lenz and coworkers [27, 28].

Group A	Support cell growth and polymer production:
	e.g. hexanoic acid to hexadecanoic acid, 5-phenylvaleric acid [26, 29-31].
Group B	Support cell growth, but not polymer production:
	e.g. 6-bromohexanoic acid, 8-bromooctanoic acid [32], 6-methyloctanoic acid [33, 34], 11-cyanoundecanoic acid [28]
Group C	No cell growth or polymer production:
	5-methyloctanoic acid [32, 33], 4-phenylbutyric acid [27]

Another interesting feature of *P. oleovorans* is the production of random copolymers by the cometabolism of mixed substrates. Cometabolism is a process whereby a 'poor' substrate, one which may not support cell growth, is utilized along with a 'good' carbon substrate, one which supports both cell growth and polymer production. The polymer synthesized may be a random copolymer with repeat units resulting from both

substrates. These poorer carbon substrates generally contain functional groups which may include halogens (bromine, chlorine, fluorine), [33-36] olefinic groups [37, 38], cyano groups [27], and methyl, ethyl, and propyl esters [39, 40].

One of the most interesting polyesters produced by *P. oleovorans* is poly-3-hydroxyphenylvalerate (PHPV), which was synthesized when *P. oleovorans* is grown on 5-phenylvaleric acid, as the sole carbon source (Figure 1.6) [33]. This was the only reported case where this bacterium has produced a homopolymer. This homopolymer has unusual properties in that it had the highest glass transition temperature, Tg, (19°C) of all the polyesters produced by *P. oleovorans* and it did not exhibit any crystallinity.

When *P. oleovorans* was grown with a mixture of 5-phenylvaleric acid (PVA) and nonanoic acid (NA), a mixture of two polymers was produced [40]. One polymer was poly-3-hydroxyphenylvalerate (PHPV) produced from the PVA, and the other contained only the long chain 3-hydroxyalkanoates of the same composition as the PHA produced with nonanoic acid alone, which is referred to in this discussion as poly-3-hydroxynonanoate (PHN). This result is unique in that all previously reported cofeeding experiments with this bacterium have produced random copolymers containing repeat units from both substrates. The substrate and polymer structures are shown in Figure 1.7.

1.2 Production of Poly-3-hydroxyalkanoates

The bacteria which produce PHA' s can be divided into two distinct groups, one which produces short-chain-length polymers (SCL-PHA's), where R = 1-2 and the other group produces medium-chain-length PHA's (MCL-PHA's), where R=3-9. The enzymes involved in the production of the short-chain-length PHA's have been isolated and studied and a number of research groups are currently involved in isolating the enzymes responsible for the production of medium-chain-length polyesters. All the evidence indicates that two distinct class's of polymerase enzymes exist. One which is present in bacteria such as *A. eutrophus* and *R. rubrum* and incorporates 3-, 4- and 5-

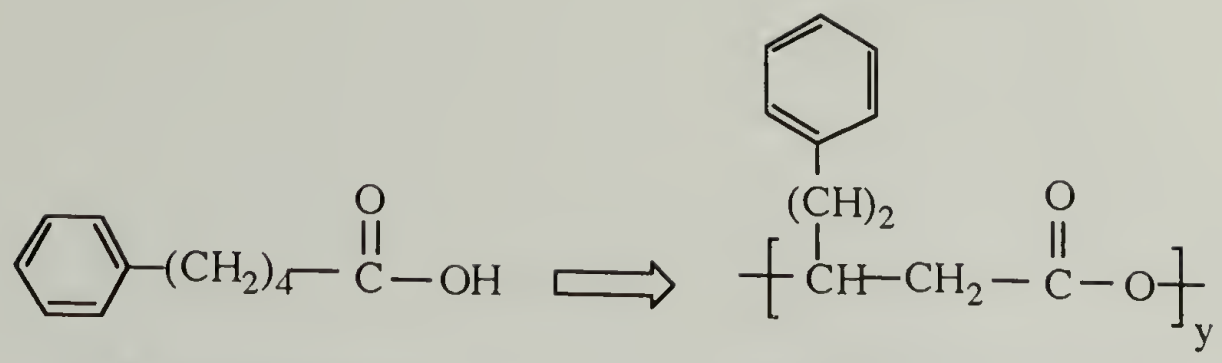
hydroxyalkanoic acids, and the other which occurs in *P. oleovorans and P. putida* which appears to be less specific in that it is capable of incorporating saturated, unsaturated, branched, and aromatic 3-hydroxyalkanoic acids of lengths between 6 and 16 carbon atoms. The general pathway of PHB production is well established [3] and shown in Figure 1.8. PHB is synthesized from acetyl-CoA by a sequence of three enzymatic reactions.

The synthesis of PHBV copolymer proceeds by the same pathway except that 3-hydroxyvaleryl is formed from propionyl-CoA and acetyl-CoA by 3-ketothiolase. The three enzymes involved in the production of PHB and PHBV have been isolated and tested for substrate specificity [41-43]. The enzymes only exhibited activity in the presence of acetyl-CoA or 3-ketopentanoyl-CoA. Only PHB was produced when the bacteria were grown on hexanoic acid, decanoic acid, decane, chloropropionic acid, and isopropanol, suggesting that the enzyme specificity of *A. eutrophus* is such that it can only accumulate polymer containing C4 and C5 repeating units.

It has also been established that the 3-ketothiolase and acetoacetyl-CoA reductase are soluble cytosolic enzymes, and PHB-synthase enzyme is located on the surface of the polymer granule [44]. The narrow substrate specificity (limited to C4 and C5 carbons) is due to the PHB polymerase enzyme.[43]. Peoples and Sinskey have isolated the three genes encoding the 3-ketothiolase (*phb*A), acetoacetyl-CoA reductase (*phb*B) and the PHB synthase (*phb*C) from *Alcaligenes eutrophus* and determined they are organized on a single operon as *phb*A-*phb*B-*phb*C [45,46].

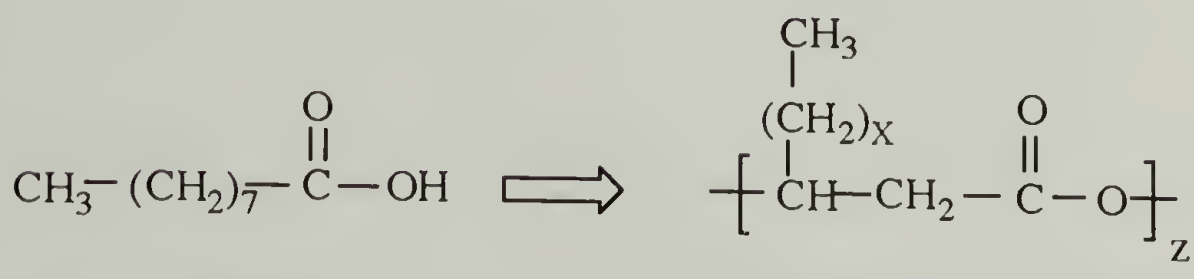
Substrate

Polymer



5-Phenylvaleric acid
PVA

Poly-3-hydroxyvalerate
PHPV



Nonanoic acid
NA

Poly-3-hydroxynonanoate
PHN

x= 3,5,7

Figure 1.7 The chemical structure of the polymers poly-3-hydroxynonanoate and poly-3-hydroxyphenylvalerate which were produced when *P. oleovorans* was grown on a mixture of nonanoic acid and 5-phenylvaleric acid

Reaction 1 Two molecules of acetyl-CoA are condensed by the action of 3-ketothiolase (*phb*A) to form acetoacyl-CoA, a free enzyme CoASH is released in the process.

Reaction 2 Acetoacyl-CoA is reduced to (R)-3-hydroxybutyryl-CoA by acetoacyl-CoA-reductase (*phb*B)

Reaction 3 PHB synthase (*phbC)* catalyses the conversion of (R)-3-hydroxybutyryl-CoA to PHB.

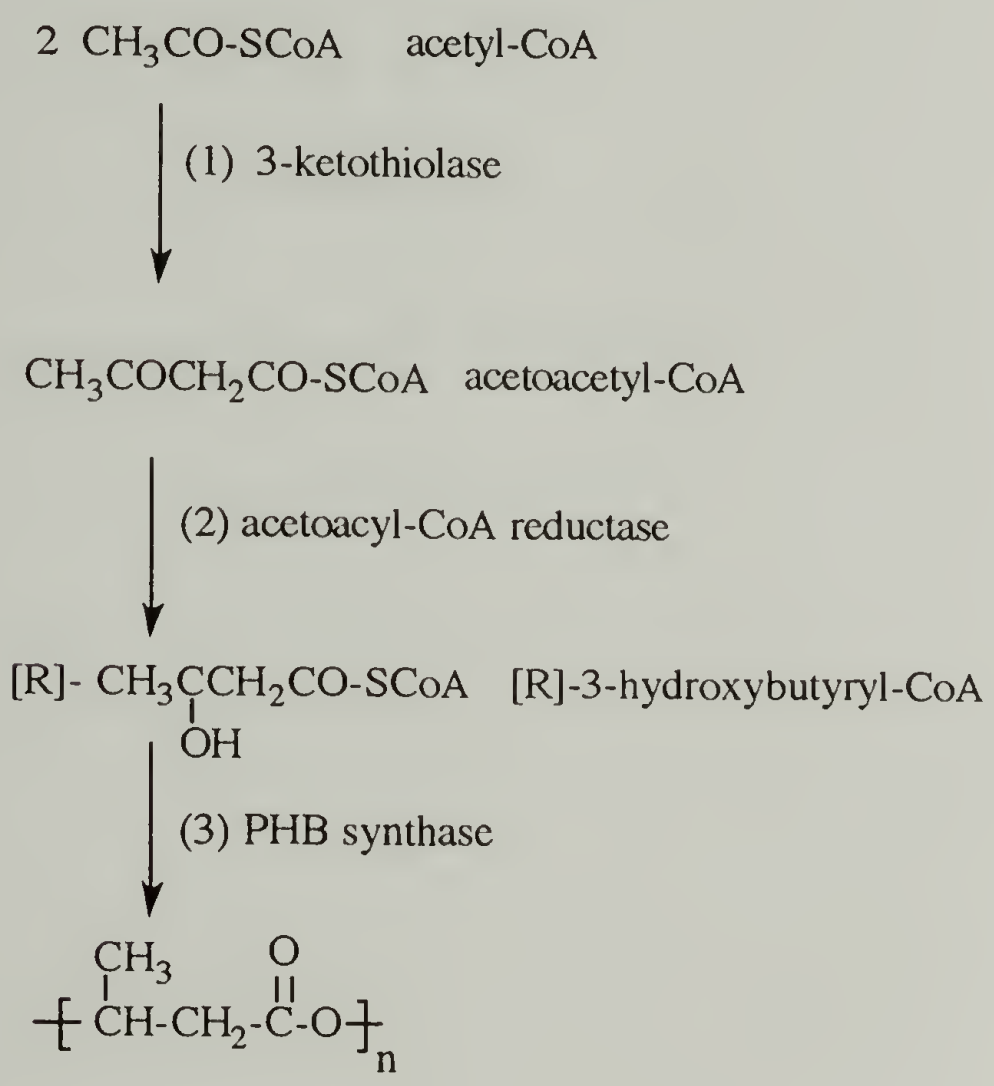


Figure 1.8 PHB synthesis pathway in *A. euthrophus*

The pathway for the synthesis of medium chain length polyesters from *P. oleovorans* is less well established, but the currently held route for the synthesis of poly-3-hydroxyoctanoate can be seen in Figure 1.9. The synthesis of PHO is more complicated than that of PHB, involving six enzymes. The polymer produced is generally a random copolymer with 3 repeating units, the main unit having the same number of carbons as the monomer and the minor units consisting of either two carbons less or two carbons more than the monomer. The formation of a random copolymer is due to the cleavage of fatty acids by the b-ketothiolase which yields an acetyl-CoA molecule and a fatty acid which is two carbons shorter than the monomer. Witholt and coworkers have carried out an in depth genetic analysis of the polymerase -depolymerase system in *P. oleovorans* , and have established the existence of two polymerase enzymes, and one depolymerase enzymes. The depolymerase enzyme is thought to be a lipase [47-49].

Haywood [50] examined 25 different bacteria known for their ability to store polyester and found that five strains accumulated PHB, 13 accumulated PHBV and 7 accumulated medium-chain-length PHA's whose repeat units ranged from 5 to 10 carbons None of the strains which were examined accumulated both short chain and medium chain length PHA's.

However, in 1992 Steinbuchel and Wiese [51] reported the discovery of a citronell-utilizing bacterium which was able to accumulate both types of polymer when grown on a range of substrates, which included glutonate or octanoate. It should bc noted that this bacteria was the only one of 45 tested which exhibited such non-specific behavior and the authors speculated that this was due to the presence of two distinct polymerase enzymes.

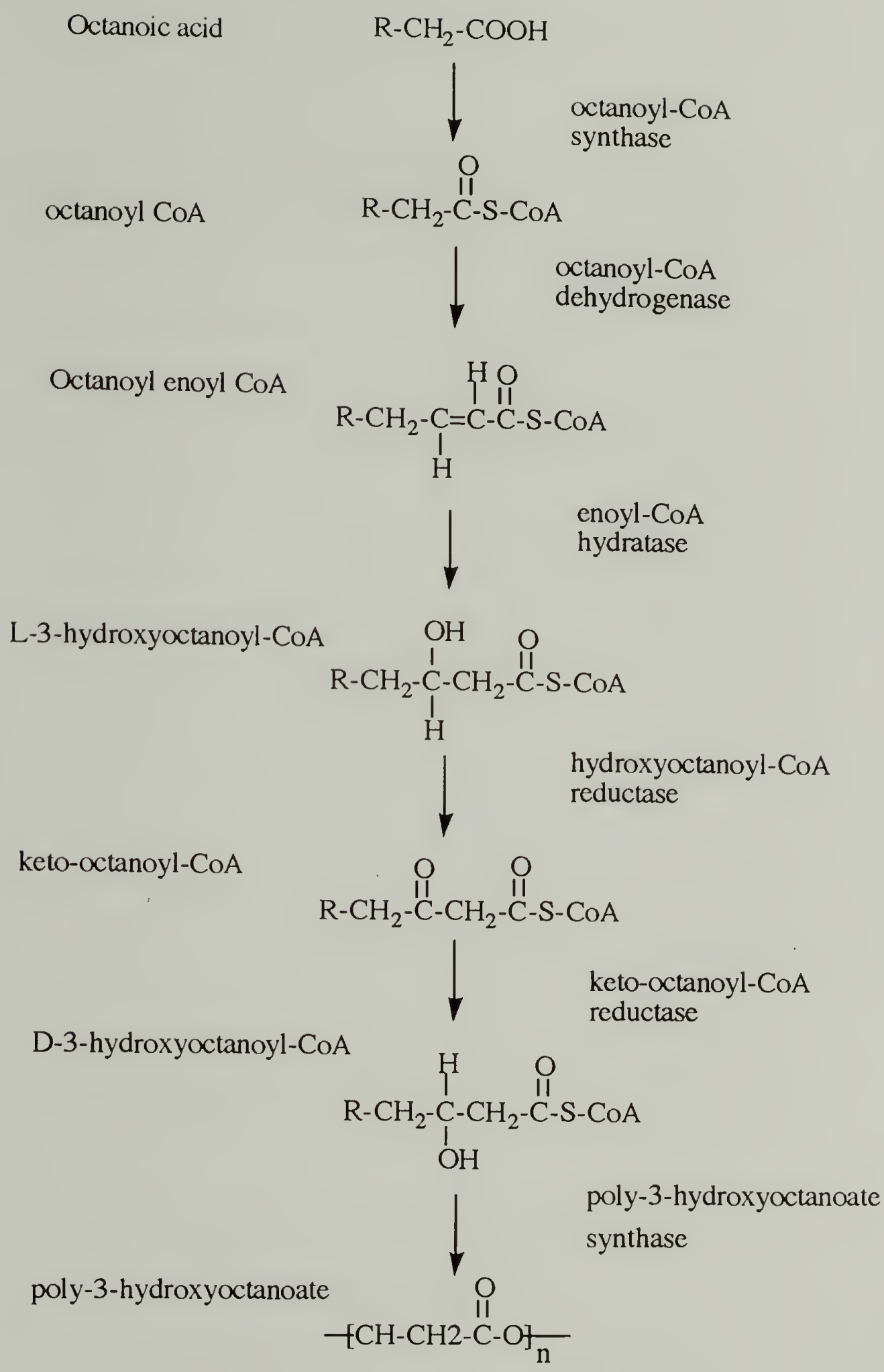


Figure 1.9 PHO synthesis pathway when octanoic acid was the sole substrate

1.3 Physical Properties of Poly-3-hydroxyalkanoates

The first PHA discovered, poly-3-hydroxybutyrate is a highly crystalline polymer (65-85% crystallinity) with a glass transition temperature, Tg, of 5°C and a Tm of 180°C. Due to its high degree of crystallinity the polymer was difficult to process. Another processing problem was due to the fact that PHB degrades thermally below 200°C leaving a small processing window. For this reason, ICI decided to commercially produce a copolymer of poly-3-hydroxybutyrate-co-valerate, using a mixture of glucose and propionic acid as carbon substrates. The commercially produced polymer, "Biopol" is available in 0-30 moles % HV, and the corresponding melting temperature vary between 180 and 110°C [52]. It was found that the incorporation of the 3-HV units into the polymer backbone decreased the degree of crystallinity and the melting point, making the polymer easier to process. Also, the copolymer was tougher and more flexible than the PHB homopolymer, leading to a wider range of potential applications.

PHB forms a 2_1 helix with two repeating units per unit cell. The copolymer PHBV also crystallizes in a 2_1 helix but with a slightly longer fiber repeat (or c dimension) of 5.96Å versus 5.56Å for PHB [52]. The copolymer exhibits the interesting phenomenon of isodimorphism: that is, the crystal lattice changes at a certain critical concentration of HV repeat units. The PHBV copolymer with a HV content of less than 30% crystallizes in the PHB lattice and copolymers with a HV content of greater than 30% crystallizes in the PHV lattice.

Medium-chain-length PHA's (MCL-PHA's) such as the polymers produced by *P. oleovorans* when grown on carbon sources of length C6-C16 also crystallize in a 2_1 helical form but with a shorter c dimension of approximately 4.45Å [53]. These polymer are less crystalline that PHB or PHBV (25% versus 60-70%). One of most crystalline MCL-PHA's, poly-3-hydroxyoctanoate is 25% crystalline. The melting points and enthalpy of melting of MCL-PHA's are tabulated in Table 1.2 [54].

Table 1.2 The melting points and enthalpies of melting for the polymers produced by *P. oleovorans* grown on carbon sources varying in length from C6 to C16 [36,29].

Carbon source	Tm °C	ΔHm cal/g
Hexanoic acid	45	
Heptanoic acid	45	1.3
Octananoic acid	56	5.34
Nonanoic acid	50	4.37
Decanoic acid	54	4.9
Undecanoic acid	45	3.92
Dodecanoic acid	46.3	4.46
Tridecanoic acid	46.3	1.91
Tetradecanoic acid	43.7	3.19
Hexadecanoic acid	47	3.68

The polymers produced by *P. oleovorans* are elastomers and so introduce another range of possible applications for these biodegradable polymers. Table 1.3 is a comparison of the physical properties of polypropylene (PP), poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyoctanoate (PHO) [55] .

Table 1.3 A comparison of the physical properties of polypropylene (PP), poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyoctanoate (PHO) [56]

Parameter	PP	PHB	PHO
Melting Point Tm (°C)	171-186	171-182	61
Glass Transition Temperature Tg (°C)	-15	5-10	-36
Crystallinity %	65-70	65-80	25
Density (g/cm3)	0.91-0.94	1.23-1.25	1.02
Molecular Weight Mw (10^5)	2.2-7	1.8	1.35
Molecular Weight Distribution	5-12	2.2-3	1.6
Flexural Modulus (GPa)	1.7	3.5-4	ND
Tensile strength (MPa)	39	40	9.3
Extension to break (%)	400	7	380
UV resistance	poor	good	good
Solvent resistance	good	poor	poor

The molecular weights of these polymers are dependent on the microorganism used and also on the carbon substrate fed to the bacteria. For instance, the molecular weight of MCL-PHA's decreases as the length of the substrate fed to the bacteria increases. Poly-3-hydroxyheptanoate has a Mw of 370,000 and poly-3-hydroxydecanoate has a molecular weight of 92,000. Also, the molecular weight of the polymer produced from *Rhodospirullum sphaeroides* varies from 370,000 to 1.5 million depending on the substrate fed to the bacteria [26].

1.4 Potential Uses of Poly-3-hydroxyalkanoates

Approximately 100 million tons of plastic is produced annually. Generally plastic materials are cheap, light, durable and inert. However, the very properties which make them attractive for the consumer market make their disposal difficult. As these products are petroleum based and chemically synthesized, they are foreign to microorganisms which for billions of years have been degrading natural polymers such as cellulose and starch. Therefore, when a synthesized plastic material is disposed of, it remains at its disposal site.

Poly-3-hydroxyalkanoates are an innovative class of materials, as they are polyesters which are produced by bacteria and so are inherently degradable. Poly-3-hydroxybutyrate has physical properties similar to polypropylene and could be used in applications where a degradable polymer is desirable. In 1990 a German company released a new shampoo "Sanara" which was packaged in a PHBV bottle.

The other class of biopolyester, MCL-PHA's are thermoplastic elastomers. In addition, the incorporation of various functional groups, such as olefinic groups, halogens, methyl ester and phenyl groups into the side-chain of these polymers could extend the field of possible applications even further.

Another attractive application of these biopolymers is their inherent biocompatibility. These polymers degrade *in vivo* by a hydrolysis procedure. The hydrolysis product of PHB, 3-hydroxybutyrate is a common metabolite in the blood, and PHB implants show a minimum inflammatory response and no rejection reactions, and therefore, would be useful as bone scaffolding. Their potential as a matrix for the controlled delivery of drugs is still being investigated. While they are biocompatible and biodegradable, the results obtained so far indicate that the *in vivo* degradation rates are too slow to be used for drug delivery. Juni and coworkers [57,58] studied the release of the drug aclarubicin from PHB microspheres and found that only 10% of the drug was released after 120 hours *in vitro,* however, the incorporation of ethyl and butyl ester of fatty acids increased the release rate. Much more research needs to be carried out before

PHA's can be used as viable drug delivery matrices. Due to the fast degradation rates of short-chain-length PHA's in soil environment, if these polymers are used for controlled delivery in the immediate future, it will most likely be to deliver pesticides and herbicides.

One reason why the biomedical applications may be more feasible than packaging applications is the cost. In the medical world the cost of a polymer is not an issue. However, in the world of low cost commodity plastics, such as those currently used for packaging and bottle manufacture, the high cost of PHB and other related PHA's (PHBV) is a pertinent issue. PHB currently costs $9/lb which is over a hundred times cheaper than it's price when it first came to market in 1980 but this still does not compare to the cost of polypropylene which is currently 20 cents/lb.

1.5 Dissertation Objectives and Outline

The objects of this dissertation were as follows:

◆ To produce PHA's from NA and PVA and to determine the intercellular location of the PHN and PHPV polymers produced.

◆ To identify the polymerase enzymes responsible for their production, in order to determine if both the PHN and PHPV polymers are produced by the same enzyme system.

◆To produce a random copolymer containing both phenylalkyl and alkyl repeat units

◆ To increase polymer yields and decrease fermentation times.

◆To produce a crystalline phenyl containing polyester, either by annealing PHPV or by growing the bacteria on carbon substrates which can produce arylalkyl units that will crystallize directly.

◆ To produce polyesters from commercially available phenylalkanes of various lengths and to determine the optimum chain length for polymer production.

◆ To study the degradation, both intracellular and extracellular of PHN and PHPV.

The procedure involved in obtaining polyesters from *P. oleovorans,* and the various techniques used in characterizing the polymers are described in Chapter 2. Chapter 3 describes the intercellular location of the PHN and PHPV polymer to determine whether they were both synthesized in the same cell and if so in the same inclusion body. The enzymes involved in the synthesis of the two polymers were isolated, and subsequently separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Also described in Chapter 3 are two separate experiments which attempted to produce a random copolymer with both phenylalkyl and alkane repeat units.

Chapter 4 describes the attempts to increase the yields of the polyesters and therefore, decrease production costs. Chapter 5 describes the various attempts to produce a crystalline phenyl-containing polyester. PHPV was annealed under varying conditions in an attempt to induce crystallinity. Also a phenyl-containing polyester was produced by feeding aromatic carbon substrates in which the para position was substituted. *Pseudomonas putida* was grown on a number of phenyl containing substrates, in order to compare its ability to store polymer with that of *Pseudomonas oleovorans.* Chapter 6 describes the fermentation experiments carried out using commercially available phenylalkane substrates. Phenylhexane through phenylnonane were fed to the bacteria to determine the optimum chain length for polymer production when the carbon substrate contains a phenyl group. Chapter 7 contains a description of the different degradation studies, both intercellular and extracellular which have been carried out on PHN and PHPV.

Finally, further experiments are outlined which could be carried out in order to gain more information on *P. oleovorans* and the intracellular polymers which it produces.

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CHAPTER 2

POLYMER PRODUCTION AND CHARACTERIZATION

2.1 Introduction

Bacterial polyesters are stored as intracellular granules as a result of a metabolic stress, which is a limited supply of an essential nutrient in the presence of excess carbon source [1,2,3]. There has been a lot of research carried out to investigate the limiting factors, such as nitrogen, phosphorus, and oxygen which result in storage of polymers. Table 2.1 contains a list of some of the bacteria known to produce intracellular storage granules and the essential nutrients, whose absence causes them to do so.

Table 2.1 Polymer-producing bacteria and the essential nutrients whose absence results in the polymer production.

Organism	Limited nutrient	Reference
Alcaligenes euthrophus	ammonia	4
Rhodospirillum rubrum	ammonia, sulfate or phosphate	5
Azobacter vinelandii	oxygen	6
Pseudomonas oleovorans	ammonia, magnesium, oxygen, phosphate, sulfate	7
Rhodobacter sphaeroides	ammonia, phosphate, sulfate	NA

As can be seen from Table 2.1, a limitation of more than one nutrient can causes the bacteria to accumulate polymer. The commercial production of PHBV by Zeneca Ltd. is a

two stage process carried out in a feed batch reactor. In the first stage, *A. eutrophus* grow and multiply in a glucose salt medium under conditions of carbon and nutrient excess [8,9]. The PHBV copolymer is produced in the second stage by feeding the bacteria a mixture of glucose and propionic acid under condition of restricted nitrogen. The restricted nitrogen condition is achieved either by allowing the nitrogen supply to become depleted or by centrifuging the cells and resuspending them in a medium which contains no nitrogen.

In addition to being a source of stored energy, there is evidence that the presence of the intracellular granules physically strengthens the cells, making them more resilient to UV radiation, and desiccation [10]. Cells containing PHA granules have a higher survival rate than cells which do not contain granules.

2.1.1 Polymer Extraction

A number of methods have been used to extract polymer from the cellular material. One method is use a solvent which lyses the cell and dissolves the polymer. Solvents which have been used for this purpose include chloroform [11], methylene chloride [12], 1,2 dichloroethane [13], and propylene chloride [14]. Another method used to separate the polymer from the cellular material is the hypochlorite method [15], where the cells are digested by sodium hypochlorite liberating the polymer. Decreases in molecular weight have been observed in cells which were treated with hypochlorite for long time periods, indicating that the polymer is being degraded. Zeneca Ltd. is currently using an enzyme treatment to separate PHPV from the *A. eutrophus* cells. The procedure involves sonicating the cells in the presence of lysozyme [16]. Doi compared the various methods of polymer isolation from *Rhodospirillum rubrum* and stated that the chloroform extraction method resulted in the highest yield of polymer and the highest molecular weights [17].

2.1.2 Polymer Characterization

The methods most commonly used to characterize these biopolymers are nuclear magnetic resonance (NMR), Wide angle X-ray diffraction (WAXS), gas chromatography (G.C.), and gel permeation chromatography (GPC). Other techniques which have been utilized less frequently are mass spectroscopy (MS) and microscopy (light microscopy, scanning and transmission electron microscopy).

Both proton (^{1}H NMR) and carbon (^{13}C NMR) have been used extensively to determine the composition of bacterial PHA's, and to prove that the copolymers produced are statistically random [17,18]. Sanders and coworkers have used ^{13}C NMR to compare the state of the polymer *in vivo* with that of the extracted crystalline polymer, and also to compare granules prepared artificially using a surfactant with those isolated from cells [19, 20]. Marchessault and and coworkers have carried out an extensive study of the crystal structure of PHA's using both NMR and Wide angle X-ray diffraction [21]

Medium-chain-length PHA's (MCL-PHA's) are usually random copolymers consisting of at least three repeat units. The major repeat unit has the same number of carbons as the monomer and the other two repeat units have either two carbon less or two carbons more due to the β-oxidation or addition of an acetyl group. Methanolysis-GC has been used extensively to determine copolymer composition [22-25]. In this characterization technique, the polymer is reacted with methanol in the presence of sufuric acid, to form the methyl esters of the corresponding repeat units, which are subsequently analyzed by G.C.. An interesting technique which has been used to prove that bacterial copolymer are statistically random is partial methanolysis of polymer to form oligomers, which are separated by HPLC and analyzed by fast atom bombardment mass spectrometry (FAB-MS) [26].

All of the polyesters discussed in this dissertation were produced by fermenting either *P. oleovorans* or *P. putida* under the appropriate conditions: that is, in the presence of an excess of carbon source and a limited supply of oxygen. The oxygen limitation was

achieved because the supply of oxygen to the shaker was constant, but as the bacteria multiplied the number of bacteria present increased, eventually resulting in a shortage of oxygen. This chapter describes the steps involved in producing bacterial polyesters and the techniques used to characterize the polymers produced. The scale of the fermentation varied, between 100mls and 12L depending on the amount of carbon source available and the amount of polymer required.

2.2 Experimental

2.2.1 Fermentation Procedure

Stock cultures of *P. oleovorans* (ATC 29347) were used throughout the course of these experiments. The strains were maintained at 4^0C on nutrient agar plates using E* media with a 10mM concentration of nonanoic acid (NA). The cultures were cultivated in a mineral modified medium, called E* medium which consisted of:

1.1g/L $(NH_4)_2HPO_4$, 5.8g/L K_2HPO_4, 3.7g/L K_2HPO_4 10 mls/L of a 100 mM solution $MgSO_4$ (in distilled water), 1.0 mls/L of a microelement solution

The microelement solution consisted of the following in 1M HCL:

2.78g/L $FeSO_4.7H_2O$, 1.67g/L $CaCl_2.2H_2O$, 0.17g/L $CuCl_2.2H_2O$, 0.29g/L $ZnSO_4.7H_2O$, 1.98g/L $MnCl_2.4H_2O$, 2.81g/L $CoSO_4.7H_2O$.

The carbon source used varied depending on the type of polyester desired. The concentration of carbon substrate added was either 10mM or 20mM. The PH was adjusted to 7.

The cultures were sterilized at 120^0C and 18lbs/sq/in for 20 minutes in an Amsco Laboratory Autoclave and then cooled to room temperature. The precultures were inoculated from a plate under sterile conditions. The growth of all cultures was monitored by measuring the optical density of the solution at 660nm with a Spectronic 20 Bausch and Lomb at a layer thickness of 1cm, referenced to distilled water. After 20-24 hours the cultures were ready to use as an innoculum for other cultures.

Fermentation of 250ml and 1L cultures were carried out in either 500ml indented Erlenmeyer or a 2.8L Fernbach flask respectively. The cultures were fermented in a Lab-line incubator shaker at 275 rpm and 30°C.

Larger scale fermentations were carried out in a 12L New Brunswick Microfermentor with an air flow of 5L/min, at a temperature of 30°C and a stirring speed of 400 rpms. The cell growth was monitored as previously described. When the cells reached the stationary growth phase, they were centrifuged in a Sorvall RC-3 centrifuge at 4000rpm for 20 minutes. The cells were then frozen and lyopholized using a Labconco Freeze Drying System at -50°C for 24-48 hours until the biomass was completely dry. The biomass was then extracted using chloroform (10x the dry cell weight) for 12 hours. The solution was filtered through a sintered glass funnel (5-20m) to remove cellular debris. The chloroform was then concentrated to 5mls using a rotary evaporator, and the polymer precipitated by dropping the chloroform into 50mls of rapidly stirring methanol. The polymer/methanol mixture was stirred for 12-24 hours, then decanted and the precipitated polymer was dried under vacuum for 24 hours.

2.2.2 Polymer Characterization

NMR spectroscopy

^{1}H and ^{13}C nuclear magnetic spectroscopy (NMR) were performed on a Bruker 200MHz and on a Varian 300MHz respectively. The temperature and magnetic field used were 17°C and 74.5 MHz. The deuterated solvents used were d-chloroform and d-acetone. The polymer concentration for ^{1}H NMR was 10mg/ml and the sample was referenced to tetramethylsiloxane (TMS). For ^{13}C NMR spectroscopy, the polymer concentration was 150mg/5ml and the sample was referenced to chloroform.

Gas chromatography

Gas chromatography (GC) was performed on a Perkin Elmer 8500 equipped with a Durabond Carbowax megabore capillary column (15m x 0.54 mm, Carrier Gas He, Flow Rate 17mls/min). G.C. was used to determine:

(a) Polymer composition and concentration

The methanolysis reaction used to convert the polymer into its corresponding 3-hydroxymethylester components, was as follows:.

6-8 mg of dry polymer was weighed and placed in a screw top vial, the cap of which was lined with teflon. 2 mls of dry chloroform and 2 mls of distilled acidified methanol (15% H2SO4) were added to the polymer. The vials placed in an oil bath at 100°C for approximately 2 hours and 20 minutes, after which 1 ml of water was added and the vial vortexed for 2 minutes. The vials were left standing to allow the two layers to separate, and the bottom chloroform layer was removed. If the sample did not separate correctly the chloroform layer was passed over Na_2SO_4. 2μl of the chloroform solution was injected into the G.C. instrument.

The G.C. instrument was calibrated using samples containing known concentrations of polymer which were prepared as described above.

The program used was as follows, the sample was maintained at 80°C for 4 minutes, heated at 8°C/min to 160°C, then held for 11 minutes before cooling to 80°C.

(b) The carbon substrate concentration of the E* media, (which was used to determine the growth state of the bacteria.)

5mls of E* media was withdrawn from the fermenting culture, and centrifuged at 11,000 rpm for 10 minutes. 2mls of this media was placed in a screw top vial, 2mls of chloroform and 200ul of H_2SO_4 was added. The vial was vortexed for 2 minutes and the left standing until the layers separated. The bottom (chloroform) layer was removed and 2µl of the chloroform solution was injected into the G.C..

The G.C. was calibrated using samples of E* media containing known concentrations of carbon substrate. The program used was one in which the sample was held at 125°C for 7 minutes, heated at 5°C/min to 200°C, then held at 200°C for 3 minutes after which it was cooled back to 125°C.

Thermal Analysis

A DuPont differential scanning calorimeter 2910 (DSC) was used to determine the glass transition temperatures (Tg's), the melting transitions (Tm's) and the enthalpies of fusion (ΔHm) of the polymers. Samples were heated from -100°C to +250°C in a nitrogen atmosphere at a rate of 20°C/min, quenched and heated a second time using the same range and heating rate. The Tg reported was the inflection temperature and the Tm was the peak temperature.

Thermogravimetric analysis was used to determine the degradation temperature of the polymers. The analysis was conducted on a TA Instruments TGA Model 2950, in a nitrogen atmosphere. Samples were heated from 30°C to 600°C at a rate of 20°C /min. The degradation temperature reported was the onset temperature of the weight loss.

Morphology

A Joel 100CX transmission electron microscope was used to examine the structure of the samples. The magnification of the sample varied between 16,000 (16KX) and 50,000 (50KX).

X-ray Diffraction

Wide angle X-Ray diffraction studies (WAXS) were carried out under reduced pressure using a Statton camera with a Siemens K710H generator operating at 40kV and 30mA. Nickle filtered CuKα radiation (λ =0.1542 nm) was used. Polymer samples of approximately 0.5mm thickness were exposed to X rays for 8 hours.

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CHAPTER 3

INTRACELLULAR STRUCTURE AND ENZYMATIC ACTIVITY INVOLVED IN THE PRODUCTION OF NOVEL POLYESTERS

3.1 Introduction

When *P. oleovorans* was grown on a mixture of 5-phenylvaleric acid (PVA) and nonanoic acid (NA), both a homopolymer and a copolymer were produced [1]. The homopolymer was poly-3-hydroxyphenylvalerate (PHPV) [2] and the copolymer contained principally 3-hydroxynonanoate units, so was referred to as poly-3-hydroxynonanoate (PHN). This result is unique because all of the previously reported cofeeding experiments with this bacterium produced only random copolymers containing repeating units from both substrates. This chapter describes the experiments which investigated the intracellular location of these two polymers to determine if both polymers occurred in the same cell or in separate cells and if the former is true did they occur in the same inclusion body or in different inclusion bodies.

The two techniques used to determine the intercellular location were as follows:

(1) Freeze Fracture Technique

P. oleovorans cells containing PHN, PHPV and a blend of PHN/PHPV were freeze fractured and the replicas examined using transmission electron microscopy (TEM) to determine if the freeze fracture patterns of PHN and PHPV were distinctive enough to differentiate between them. This technique would only work if the two polymers were stored in different inclusion bodies and if their freeze fracture diffraction patterns were distinctive.

(2) Ruthenium Tetraoxide Staining

P. oleovorans cells containing PHN, PHPV and a blend of PHN/PHPV were fixed and stained with ruthenium tetraoxide (RuO_4), a preferential stain for phenyl groups [3], examined by TEM.

Freeze fracture is a technique where a specimen is rapidly frozen, and fractured with a cold knife at temperatures between -270 and -100^0C. The fracture often follows a plane of weakness through the specimen, which is commonly the line of a cell membrane. The schematic of the freeze fracture of a polymer granule is shown in Figure 3.1. This fracturedspecimen is then coated twice, initially with platinum and then with carbon. The bacterial sample underneath is etched away using acid and/or bleach, leaving behind a replica of the bacterial sample which can be viewed by transmission electron microscopy. Also, by varying the angle at which the stage is viewed a three dimensional picture of the samples can be obtained

When a polymeric inclusion is freeze fractured the result is either a 'mushroom' or a 'spike' shape. Both the short chain polyester, poly-3-hydroxybutyrate (PHB) and the medium chain length polymer, poly-3-hydroxyoctanoate (PHO) have been freeze fractured. Two different freeze fracture patterns were obtained. When PHB granules in *Bacillus cereus* were freeze fracture at -100^0C, a spike shape was produced [1], and when PHO was freeze fractured at the same temperature a mushroom shape was obtained [4]. The two different theories which have been put forward to explain the origin of these two distinct freeze fracture patterns are as follows [5]:

(I) The first hypothesis is that the fracture pattern is a function of the degree of crystallinity in the polymer sample. The more amorphous a sample is, the greater its elastic component and the more it will be inclined to retract back after fracturing, hence the mushroom shape. The more crystalline sample will retract less, hence the spike deformation. Extracted PHB is a highly crystalline polymer (60-70%) whereas PHO is less

crystalline (25-30%) so the amorphous nature of the PHO polymer may allow it to retract more readily. It may have a greater elastic component, resulting in elastic deformation

(II) The fractured shape is a result of the temperature of fracture, that is, it depends on how much lower than the T_g the fracture is carried out. The greater the temperature difference the lower the amount of energy available to the polymer resulting in a reduction in the number of spikes occurring. The Tg's of the extracted polymers are very different. The Tg of PHB is 5°C and the Tg of PHO is -36°C, so at -110°C it is possible that the PHO is less glassy than the PHB and retracts more than the PHB polymer, resulting in a mushroom shaped freeze fracture pattern.

The first hypothesis can be discarded as it has been proven by a number of groups working independently that the *in vivo* polymer is an amorphous elastomer [6-8] so the degree of crystallinity of the extracted polymer is irrelevant to the freeze fracture patterns of polymer granules *in vivo*.

The second method used to determine the intracellular location of the two polymers involved staining samples with ruthenium tetraoxide (RuO_4), a preferential stain for phenyl groups. Prior to staining the bacterial samples were 'fixed'. This involves replacing the water in the cell with an epoxy (generally Spur™) which is subsequently cured. The samples were then cut into 900Å thin section and examined using transmission electron microscopy (TEM) [2]. Figure 3.2 shows a micrograph of *Rhodospirillum rubrum* cells which contained intracellular inclusion granules of poly-3-hydroxybutyrate, magnified 30,000 times. Close examination of these bacterial cells reveals both gray and white areas. The gray areas represent the PHB polymers and the white areas are locations where the polymer was dissolved and replaced by epoxy during the fixation procedure. Because of the solubility of the polymer in 'Spur'™ epoxy, this commonly used fixation procedure cannot be used if one wishes to distinguish between two intracellular polymers. For this reason, a new fixation procedure had to be devised, one in which neither polymer dissolved in the chemicals or in the epoxy which is used to fix the cells.

A similar situation to the intracellular location of PHN and PHPV was investigated by Timms and coworkers [9], who reported the production of PHB and PHO in the same cell using a recombinant strain of *P. oleovorans* which harbored the poly-3-hydroxybutyrate-biosynthesis genes of *Alcaligenes eutrophus*. The bacteria accumulated up to 70% of the cell dry weight as polymer when grown on sodium octanoate as a carbon source. Approximately one half of the polymer produced was PHB. Dynamic scanning calorimetry, gel permeation chromatography, separation based on density and solvent fractionation proved that the two polymers formed a blend rather than a block copolymer. Freeze fracture analysis was used to determine the intracellular location of the two polymers [10]. Preusting and coworkers determined that the two polymers were stored in separate granules which occurred in the same cell.

In addition to determining the intracellular location of the two polymers, the role of the polymerase enzymes in the production of PHN and PHPV was also investigated. Early work by Merrick and coworkers [11] showed that the PHB biosynthesis enzyme was located on the granule surface, most likely associated with the granule membrane. Therefore, it was most likely that the *P. oleovorans* polymerase enzyme is also located on the granule surface, which has since been proven by Stuart and coworkers [12]. Huisman and Withold [13, 14] have performed a thorough genetic analysis on the polymerase-depolymerase system in *P. oleovorans*. The granules were separated from the cellular material and the enzymes isolated from the granules and separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The main bands observed occurred at 56, 54, 43, 32 and 18 KDa. It was determined by N terminal sequencing and the amino acid sequencing that the bands at 54 and 56 KDa could be assigned to the two polymerase enzymes of *P. oleovorans* The two polymerase enzymes are 53% identical and show 35-40% identity with the poly(3-hydroxybutyrate) polymerase. The 56KDa polymerase exhibits a slightly higher affinity for the 3-hydroxyhexanoate monomer compared to the lower molecular weight polymerase. From genetic data and

complementation studies the band at 32KDa has been determined to be a lipase which is the PHA depolymerase enzyme. The band which occurs at 43KDa has been assigned as being a structural protein and the band at 18KDa may be a breakdown product of this band.

The aim of the work described in this chapter was to identify the polymerase enzymes responsible for the production of the unique PHPV polymer and to determine if PHPV was synthesized by the same polymerase enzymes which synthesize PHO and PHN. Another possibility was that there was another separate, previously undetected, polymerase enzyme which produced the phenylalkyl-containing polyester.

The final section of this chapter describes two separate experiments to produce a random copolyester containing both phenyl and alkane sidechains by varying (1) the growth conditions and (2) the length of the alkane sidechain

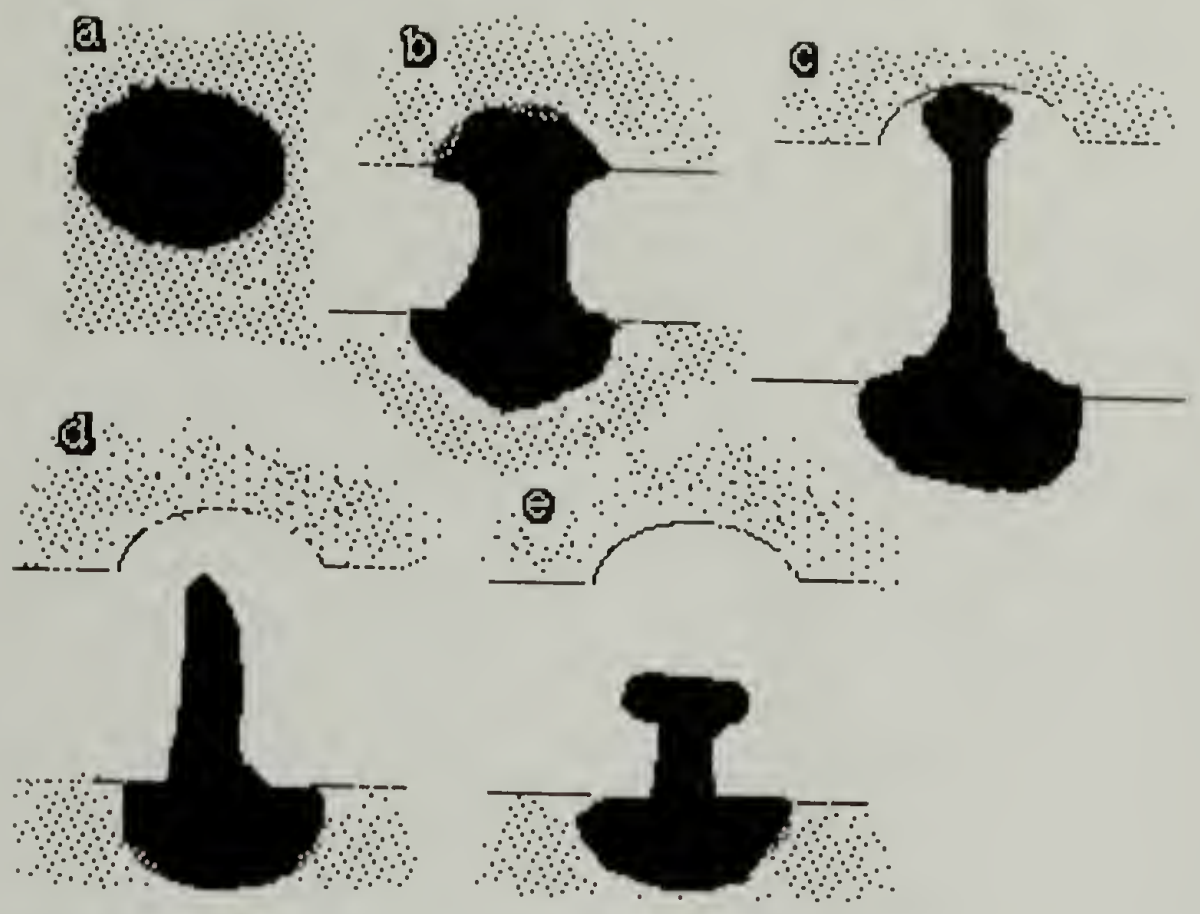


Figure 3.1 A schematic representation of the systematic fracture of a PHA granule [5]. (a) initially the granule is stretched and (b-d) one half of the granule remains embedded in the surrounding matrix, while the upper half is stretched and eventually breaks away from the upper section of the matrix; (e) the granule may retract resulting in the formation of a mushroom shape.

3.2 Experimental

3.2.1 Intraeellular Location of PHN and PHPV

3.2.1.1 Freeze Fraeture

Stock cultures of *P. oleovorans* (ATCC 29347) were used in these experiments. *P. oleovorans* was grown on 20mM of carboxylie aeids aeeording to the method in Seetion 2.2.

250 ml cultures of the following samples were prepared:

P. oleovorans grown on NA whieh produeed PHN

P. oleovorans grown on PVA whieh produeed PHPV

P. oleovorans grown on an equimolar mixture of NA and PVA whieh produced a blend of PHN and PHPV.

Samples were withdrawn periodically and the optieal density measured at 660nm. The cells were harvested when the optieal density was no longer inereasing, indicating that the culture was in the stationary growth phase. The eultures were centrifuged at 4000 rpm for 20 minutes and 5μl of the slurry was transferred into a eup, plaeed in liquid propane for 2-3 seeonds, then stored in liquid nitrogen. The samples were fraetured at -100^{0}C and etched at -170^{0}C for 4 minutes. The temperature was then returned to -100^{0}C, at whieh point the samples were sputter coated with 1nm of platinum and 20nm of carbon. The samples were removed from the freeze fraeture unit and washed in a 20% bleach solution, and then in deionized water after which the replicas were viewed using Philips CM 20 electron microscope. All the negatives of the freeze fracture speeimens have been reversed so that the shadows appear black.

3.2.1.2 TEM Sample Preparation

'Nanoplast ', 'Spur', and 'Durcupan' were purchased from Electron Microscopy Sciences. The procedure for growing the cultures is the same as in the previous section

250 ml cultures of the following samples were prepared:

P. oleovorans grown on NA which produced PHN

P. oleovorans grown on PVA which produced PHPV

P. oleovorans grown on an equimolar mixture of NA and PVA which produced a blend of PHN and PHPV.

When the cultures had reached the stationary growth phase 125mls of culture was withdrawn and 125mls of a gluteraldehyde buffer added. The buffer consisted of 5% gluteraldehyde, 0.05 M PO4, 2% sucrose, 1% tannic acid at a pH of 7. The mixtures were centrifuged at 4000rpm for 20 minutes, and the cell pellets embedded in 1% agar in 0.1 M sodium cacodyl, covered with gluteraldehyde buffer for 2 hours and cut into small pieces. These small pieces were washed with deionized water, placed in a solution of 1% uranyl acetate for 30 minutes, and then exposed to 'Nanoplast' for 1 hour. This was replaced by 'Nanoplast' and catalyst (0.25g/10g of Nanoplast) for 15 hours after which it was embedded in a flat polyethylene embedding mold and cured at 40°C for 48 hours and 60°C for 24 hours. The samples were sectioned into 900Å thick sections, placed on gold grids, exposed to ruthenium tetraoxide (RuO_4) vapor for 1 hour, after which they were viewed using a Joel CX100 transmission electron microscope

3.2.2 Polymerase Enzyme Isolation

P. oleovorans was grown on 20mM of carboxylic acids and samples were prepared by the following procedures:

P. oleovorans grown on NA in a 4L vessel

P. oleovorans grown on PVA in a 10L vessel

P. oleovorans grown on an equimolar mixture of NA and PVA in a 4L vessel

In each case 5 ml samples were withdrawn periodically and the optical density was measured at 660nm. The concentration of carbon source in the media was monitored using gas chromatography. The cells were harvested when the optical density was no longer increasing and the concentration of carboxylic acid in the media was at a minimum indicating that the cells were in the stationary growth phase. The granule isolation and purification were performed according to the method of Fuller and coworkers [15] with the following modifications.

After rupturing the cells using a French Press, 1mg/ml of PMSF and 10mM of EDTA were added to the cells and stirred gently for 30 minutes, and the solution was centrifuged at 6500rpm for 10 minutes. The supernate was separated from the cell debris and was layered on 50% glycerol gradient and then centrifuged at 9500 rpm for 30 minutes. The polymer granule complex was then recovered from the glycerol supernate interface diluted with 2mls of water and centrifuged at 17,000 rpm for 10 minutes. The enzymes were released from the granule using 1µl/ml of SDS. 15µl aliquots of the enzyme solution was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The gel was stained for 1/2 to 1 hour using a Coomassie blue staining solution 1L of which is made up as follows:

Methanol 400mls, Acetic acid 100 mls, Coomassie blue 2.5g, Distilled water 500mls

Then the gel was destained over a period of a couple of hours using Coomassie blue destaining solution, 1L of which is made up as follows:

Methanol 400mls, Acetic acid 100 mls, Distilled water 500mls

3.2.3 Experiments to Produce Random Copolymers

3.2.3.1 By changing the Growth Conditions

The bacteria was initially grown on PVA as the sole carbon substrate, than after 30 hours, when the optical density had reached 0.3 the cells were centrifuged at 4,000 rpm for

20 mins. and resuspended in E* media containing an equimolar mixture of NA and PVA. The culture was harvested after a total of 62 hours when a maximum optical density (O.D.) of 2.3 was achieved. The polymer was extracted and characterized

3.2.3.2 By varying Alkane Carbon Chain length

12L of *P. oleovorans* was grown on an equimolar mixture of 5-phenylvaleric acid (PVA) and hexanoic acid (HA). The cell yield, polymer yield and % polymer (based on dry cell weight) are shown in Table 3.1. When the maximum O.D. had been achieved, the cells were centrifuged, extracted, and the resulting polymer characterized and fractionated.

3.2.3.3 Characterization of the polymers produced

The polymer obtained from the two experiments above was fractionated to determine whether it was a copolymer or a mixture of two polymer. While both PHN and PHPV are soluble in chloroform, PHPV is insoluble in hexane. 0.5 g of polymer was dissolved in rapidly stirring chloroform and hexane was added until the solution became cloudy. The solution was then centrifuged at 13,000 rpm for 15 minutes and the hexane insoluble polymer removed. Both fractions were vacuum dried and characterized using ^{1}H NMR spectroscopy.

The polymers were characterized using ^{1}H NMR, DSC, GC and TEM. Details of the characterization procedures used are in Section 2 2.

3.3 Results and Discussion

3.3.1 Intracellular Location of PHN and PHPV

3.3.1.1 Freeze Fracture

Figure 3.2 a and b show the freeze fracture micrographs of *P. oleovorans* containing PHN and PHPV respectively. Of the numerous freeze fracture patterns examined the only pattern observed was that of spikes. The only difference between the freeze fracture patterns of PHN and PHPV was that the patterns of PHPV were lighter due the presence of PHPV underneath the replica. It appeared that PHPV was more resilient to digestion by bleach.

3.3.1.2 Ruthenium Tetraoxide Staining

Figure 3.3 is a photomicrograph of *Rhodospirillum rubrum* cells which contained inclusion bodies of poly-3-hydroxybutyrate (PHB), magnified 30,000 times. The fixation procedure in this case resulted in the dissolution of large portions of the granules, as indicated by the presence of the white areas. The grey regions inside the cell are inclusion bodies which remained intact. Therefore, a major requirement for this TEM measurement was the necessity to implement a procedure other than the commonly used embedding techniques in order to prevent the dissolution and subsequent replacement of the polymers by epoxy. The most commonly used embedding techniques are those which involve using either ethanol or Durcupan™ to dehydrate the cell. This is then replaced with Spur™ epoxy which was subsequently cured. PHPV polymer was soluble in both Durcupan™ and the Spur™ epoxy resulting in the replacement of the polymer by the epoxy. Ethanol cannot be used to dehydrate the cell due the possibility of an explosive reaction on contact with RuO_4. Nanoplast™ a water soluble melamine formaldehyde resin, was found to be a viable alternative as neither polymer dissolved in it. Also it eliminated the need for a dehydrating step as the Nanoplast™ itself is water soluble. The result provided a more complete picture of the intracellular situation. The electron micrographs of cells of *P.*

oleovorans containing PHN, PHPV and a mixture of PHN and PHPV, which were stained with RuO_4 are shown in Figure 3.4-3.6. The following observations were made:.

(1) the PHN inclusion bodies did not stain,

(2) the PHPV inclusion bodies did stain,

(3) when the cells were grown on an equimolar mixture of NA and PVA the observed pattern was one in which the core of the polymer was unstained while the outer layer of the granule was stained. Therefore, it was concluded that the polymer in the inclusion core was PHN while the surrounding polymer was PHPV. This result indicates that the two polymers were formed sequentially within the same granule.

This result compliments previous work carried out by Kim and coworkers [3], in which *P. oleovorans* was fed an equimolar mixture of NA and PVA. The E* media was sampled periodically to determine the rate of consumption of the alkanoic acids. The results showed that the substrates were utilized sequentially, with 60% of the NA being used prior to any consumption of the PVA (Figure 3.7).

3.3.2 Polymerase Enzymes which Produce PHN and PHPV

The enzymes involved in the production of poly-3-hydroxyoctanoate, (PHO), have been separated and isolated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) by Fuller and coworkers [15]. Two polymerase bands were observed, one occurred at 59KDa and the second at 55KDa. By adapting this procedure slightly, *P. oleovorans* was grown on NA, PVA and an equimolar mixture of NA/PVA, and in each case the polymer granules were isolated and the enzymes involved in polymer production were removed from the granules and separated using SDS-PAGE. Figure 3.8 is a photograph of the SDS-PAGE obtained when the enzymes were isolated from granules. The proteins in each lane were obtained from the granules of the following polymers: Lane 1 from PHO, Lane 2 from PHN, Lane 3 from PHN/PHPV and Lane 4 from PHPV.

Lane 1, which contained the enzymes isolated from the PHO granules was used as a standard because this is the inclusion body that both Withold and coworkers and Fuller and coworkers have studied most intensively. The polymerase bands at 59 and 55KDa, which were previously reported for the PHO granules, were evident in all of the four lanes. So in the case where PVA was the sole carbon source the two previously detected polymerase bands at 55 and 59 kDa were present and there was no evidence of the presence of any previously undetected bands. This observation suggests that it is most likely that PHPV polymer was synthesized by the same enzyme system which produced the PHN and PHO polymers.

Throughout the course of this experiment the concentration of carbon substrate in the E* media was monitored using G.C., and the bacteria was harvested when the concentration of carbon substrate was a minimum (> 0.05 mM) because the presence of a substantial amount of nonanoic acid interfered with the protein band separation in SDS-PAGE.

3.3.3 Attempts to Produce Random Copolymers

3.3.3.1 By Varying Growth Conditions

The growth curves obtained when NA and PVA were used as the sole carbon source are shown in Figure 3.9. NA was a good carbon source, in that the culture reached a maximum O.D. of 3 in 24 hours and a polymer yield of 1g/L was obtained. PVA was a poorer carbon source. A maximum O.D. of 1.5 was obtained after 70 hours and 100mg/L of polymer was obtained.

As the two polymers were produced by the same enzyme system which preferentially utilized NA, the 'good' substrate, the question to be answered was whether it would be possible to have the bacteria utilize both substrates at the same rate by changing the growth conditions. *P. oleovorans* was initially grown on PVA as the sole carbon source, then when the lag time was overcome (that is when the O.D. reached 0.3 after 30

hours), the culture was centrifuged and resuspended in media which contained an equimolar mixture of NA and PVA. The concept being that once the bacteria were accustomed to accumulating PHPV it might be able to metabolize both substrates at the same rate resulting in a random copolymer. Figure 3.10 shows the growth curve for this experiment which attempted to produce a random copolymer with repeating units from both NA and PVA.

The DSC thermogram of the resulting polymer (Figure 3.11) exhibited two glass transition temperatures, (Tg's), indicating that the polymer produced was a mixture of two polymers. The Tg at -28 °C corresponded to that of PHN while the one at +9°C corresponded to that of the PHPV polymer. In addition, the polymer produced was separated into its components by solvent fractionation. Initially the mixture consisted of 52% PHPV. The two fractions were analyzed by ^{1}H NMR (Figure 3.12) which revealed that the hexane insoluble fraction was 84% PHPV while the hexane soluble fraction contained 25% PHPV units. This result confirmed that the polymer obtained by changing the growth condition was not a random copolymer but was an immiscible blend of two polymers.

3.3.3.2 By Adjusting the Carboxylic Acid Chain Length

In order to eliminate the possibility that two polymers were formed because of steric hindrance, (that is, that the phenyl containing repeat unit was too large to fit between two aliphatic repeat units) the following experiment was performed:

P. oleovorans was grown on an equimolar mixture of 5-phenylvaleric acid and hexanoic acid (HA). Therefore, the predominant aliphatic repeat unit consisted of a 3 carbon sidechain instead of the 6 carbon sidechain which was formed when NA was used as the feed source. If adjacent repeating unit packing was the problem then the replacement of NA with the shorter HA might eliminate that problem and permit the formation of a random copolymer

The DSC thermogram (Figure 3.13) of the polymer obtained when the bacteria were cofed an equimolar mixture of HA and PVA exhibited two glass transition temperatures, (Tg's), indicating that the polymer was a mixture of two polymers. The Tg at -31 ^{o}C corresponded to that of poly-3-hydroxyhexanoate (PHH) while the one at +18^{o}C corresponded to that of PHPV. In addition, the polymer was separated into its component polymers by solvent fractionation. The polymer blend originally contained 50% PHPV. The two component fractions were analyzed by ^{1}H NMR, (Figure 3.14) revealing that the hexane-insoluble fraction contained 88% PHPV while the hexane-soluble fraction contained 29% PHPV units. This result confirmed that decreasing the length of the alkane sidechain did not result in the formation of a random copolymer but in an immiscible mixture of two polymers.

The cell yield, polymer yield and the % polymer (based on dry cell weight) obtained in these two experiments to produce a random copolymer are summarized in Table 3.1.

(a)

(b)

Figure 3.2 (a) Transmission electron micrograph of the freeze fracture replicas of cells of *P. oleovorans* containing PHN granules, magnified 72,000 times
(b) Transmission electron micrograph of the freeze fracture replicas of cells of *P. oleovorans* containing PHPV granules, magnified 72,000 times

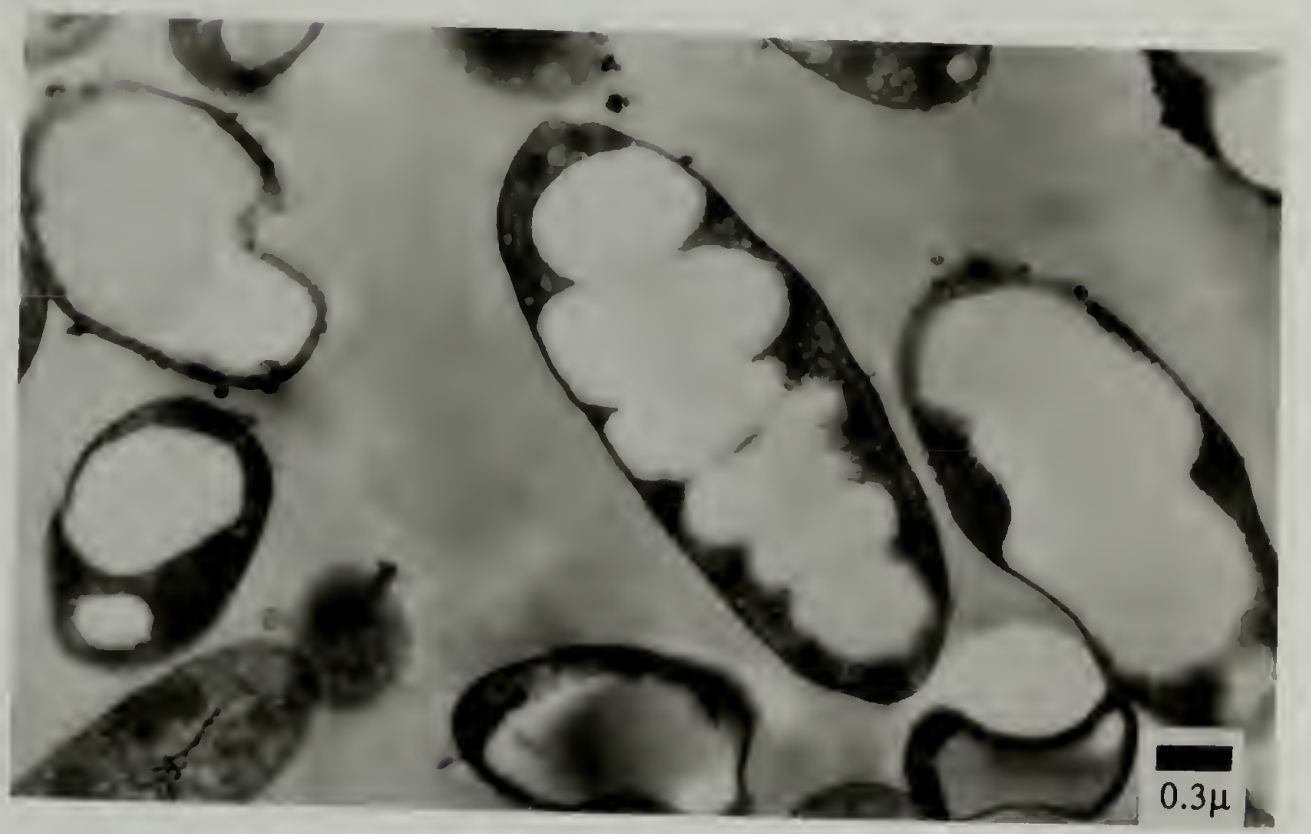
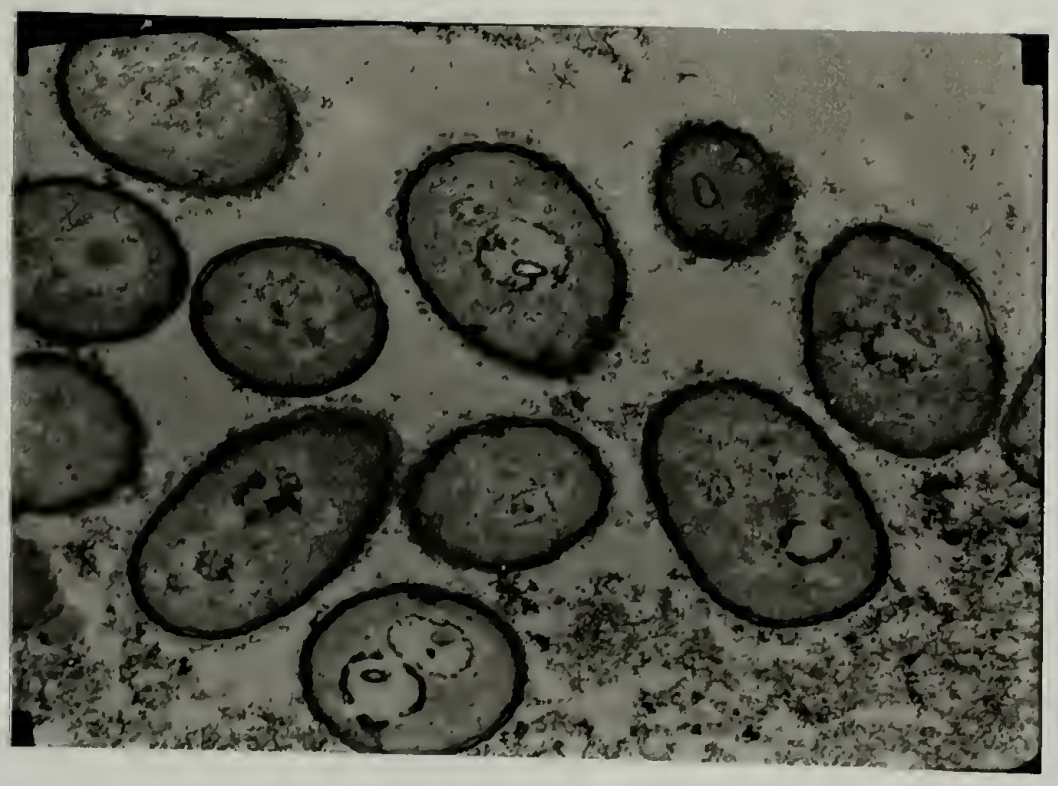


Figure 3.3 Transmission electron micrograph of *Rhodospirillum rubrum* cells containing granules of poly-3-hydroxybutyrate (PHB), magnified 30,000 times.(Micrograph courtesy of E Knee Jr., University of Massachusetts)

(a)



(b)

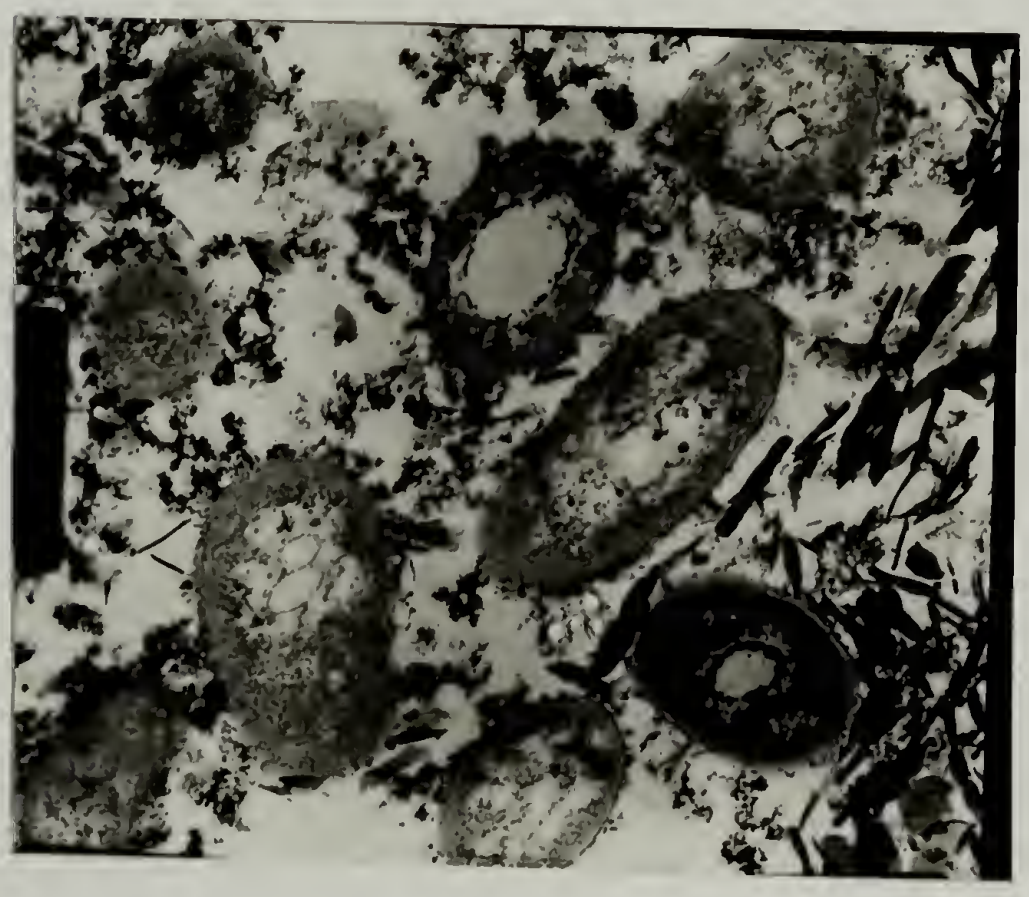
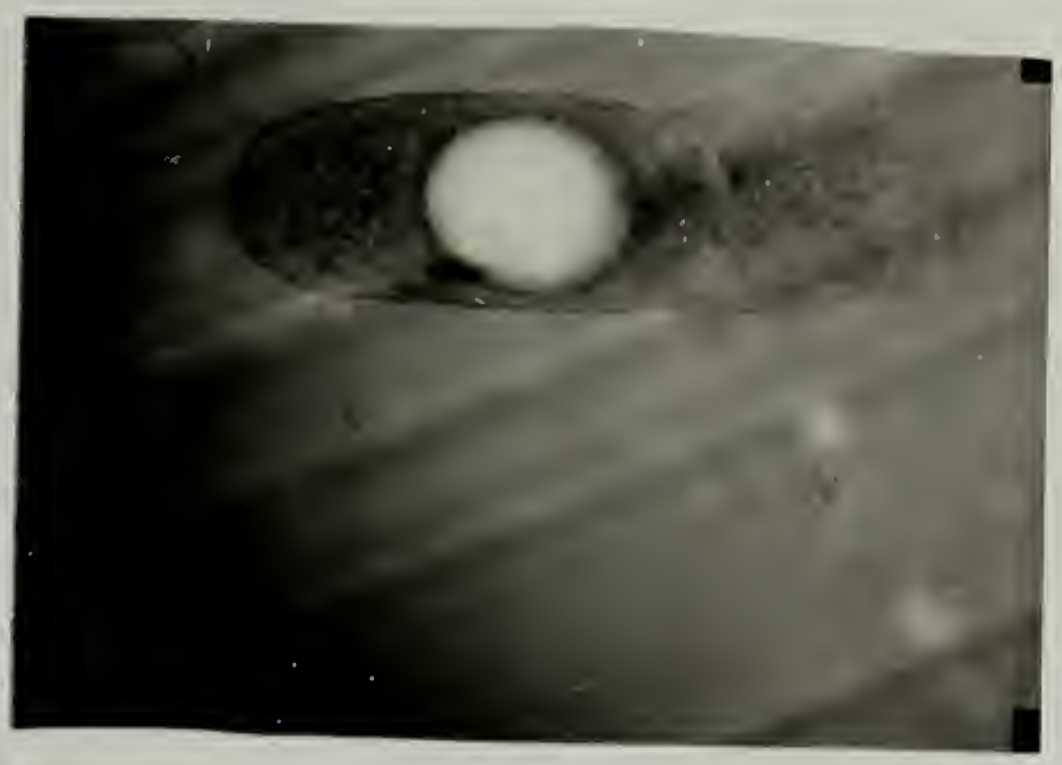


Figure 3.4 (a) Transmission electron micrograph of *P. oleovorans* cells containing unstained PHN, magnified 28,500 times.
(b) Transmission electron micrograph of *P. oleovorans* cells containing PHN, stained with RuO_4 for 1 hour, magnified 20,000 times.

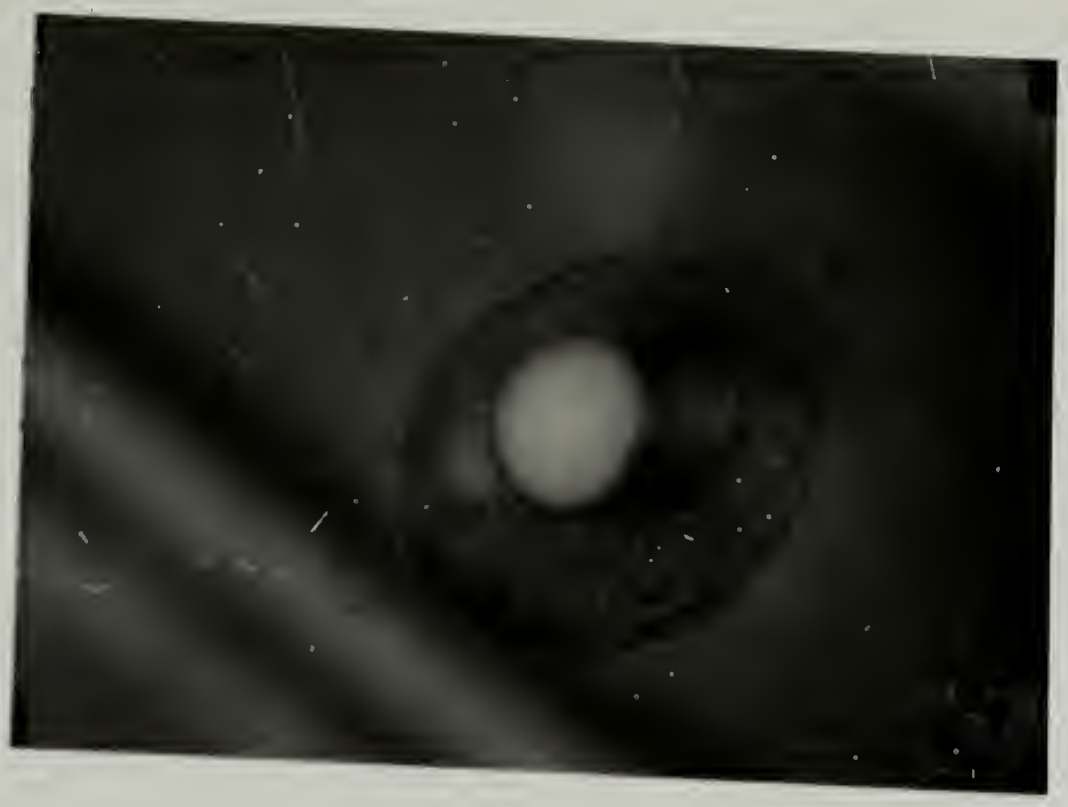
(a)



(b)

Figure 3.5 (a) Transmission electron micrograph of *P. oleovorans* cells containing unstained PHPV, magnified 33,000 times.
(b) Transmission electron micrograph of *P. oleovorans* cells containing PHPV stained with RuO_4 for 1 hour, magnified 26,000 times

(a)



(b)

Figure 3.6 (a) Transmission electron micrograph of *P. oleovorans* cells containing a mixture of PHN and PHPV unstained, magnified 50,000 times.
(b) Transmission electron micrograph of *P. oleovorans* containing a mixture of PHN and PHPV stained with RuO_4 for 1 hour magnified 50,000 times.

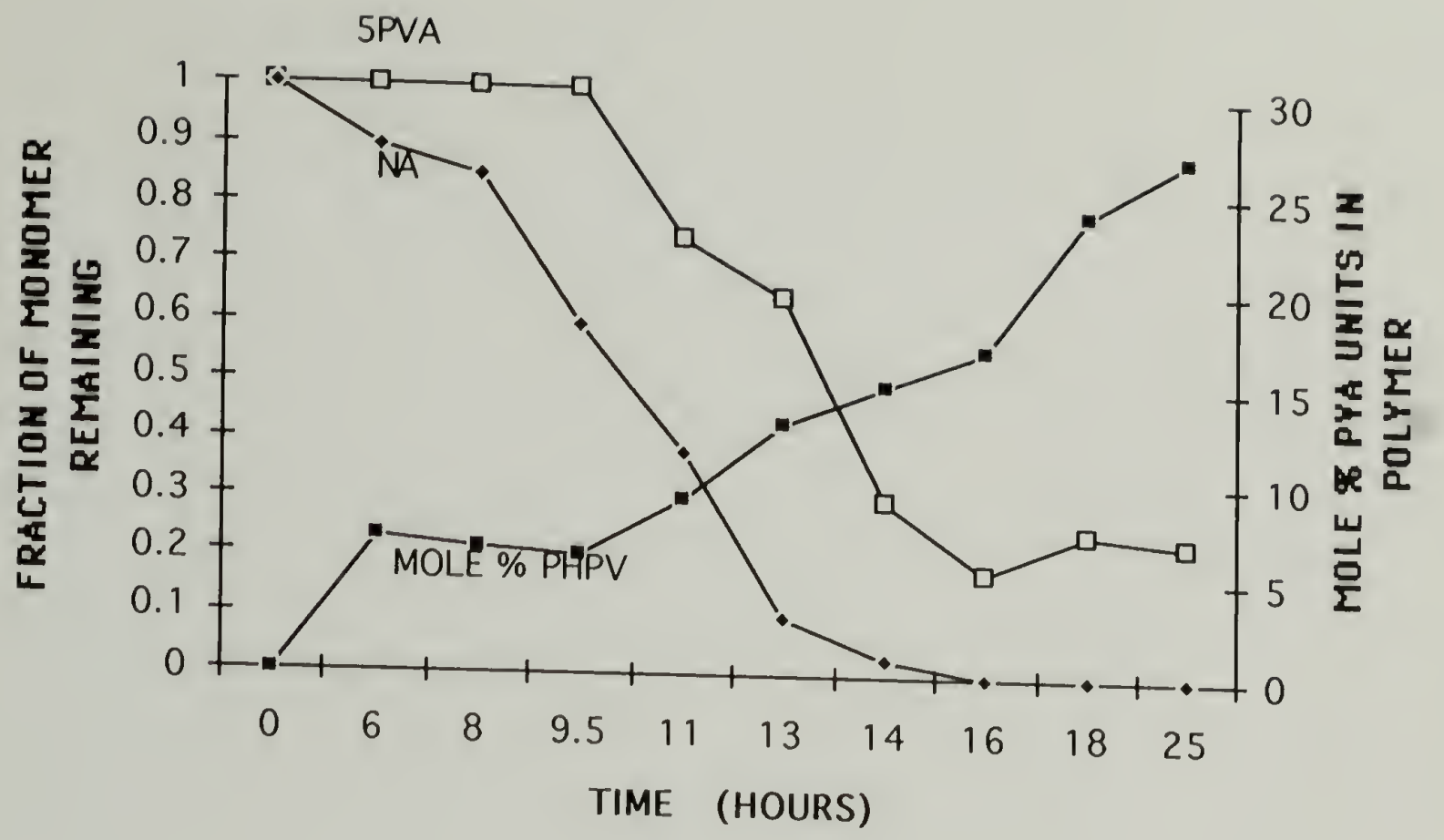


Figure 3.7 The fraction of monomer remaining and the mole % phenyl repeating units in the polymer as a function of time [1].

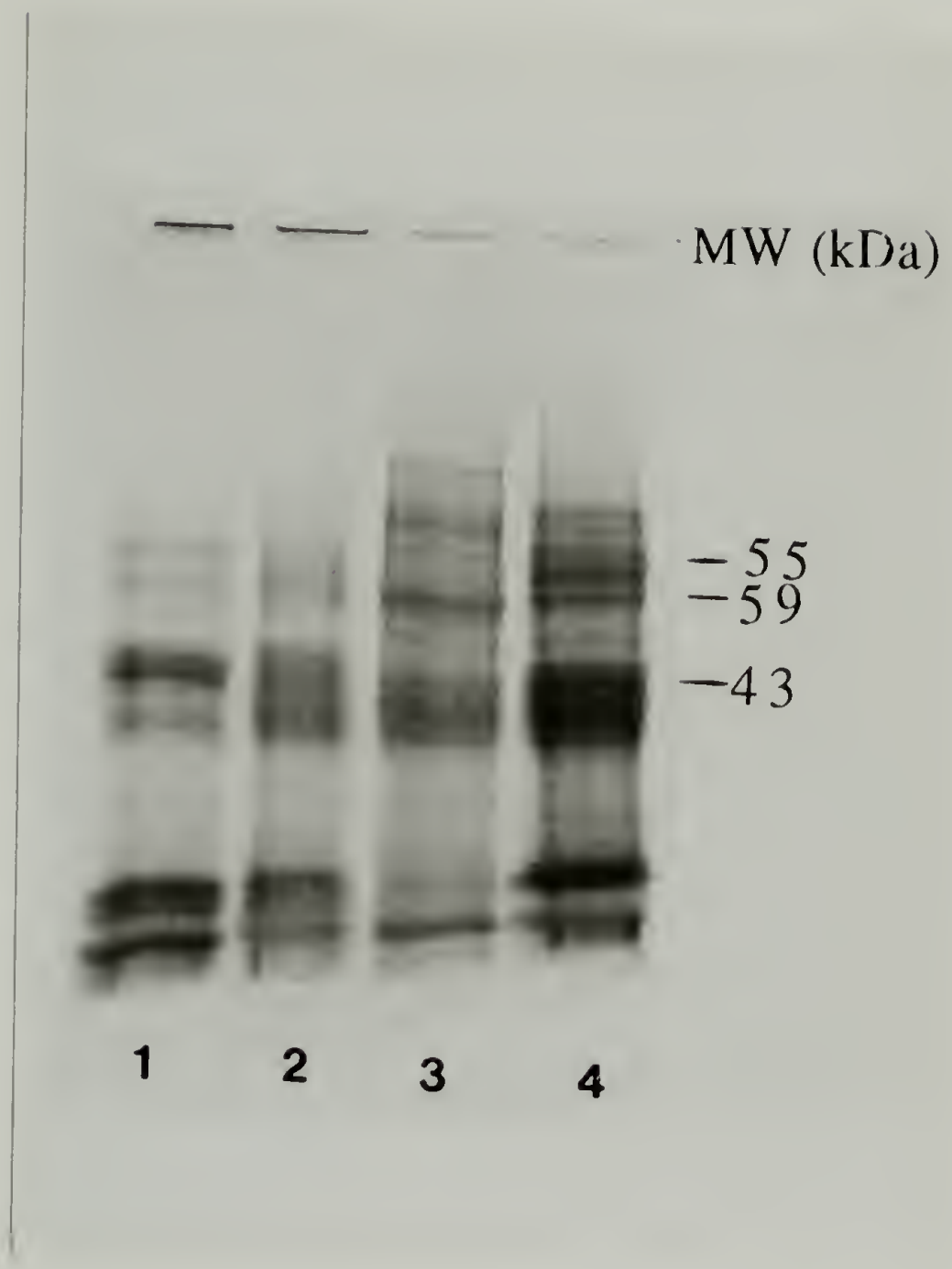


Fig. 3.8 SDS-PAGE gel of the enzymes isolated from *P. oleovorans* grown on the following substrates: lane 1 on octanoic acid (OA) to produce PHO granules
lane 2 on nonanoic acid (NA) to produce PHN granules
lane 3 on an equimolar mixture of NA and PVA to produce a mixture of both PHN and PHPV.
lane 4 on 5-phenylvaleric acid (PVA) to produce PHPV granules

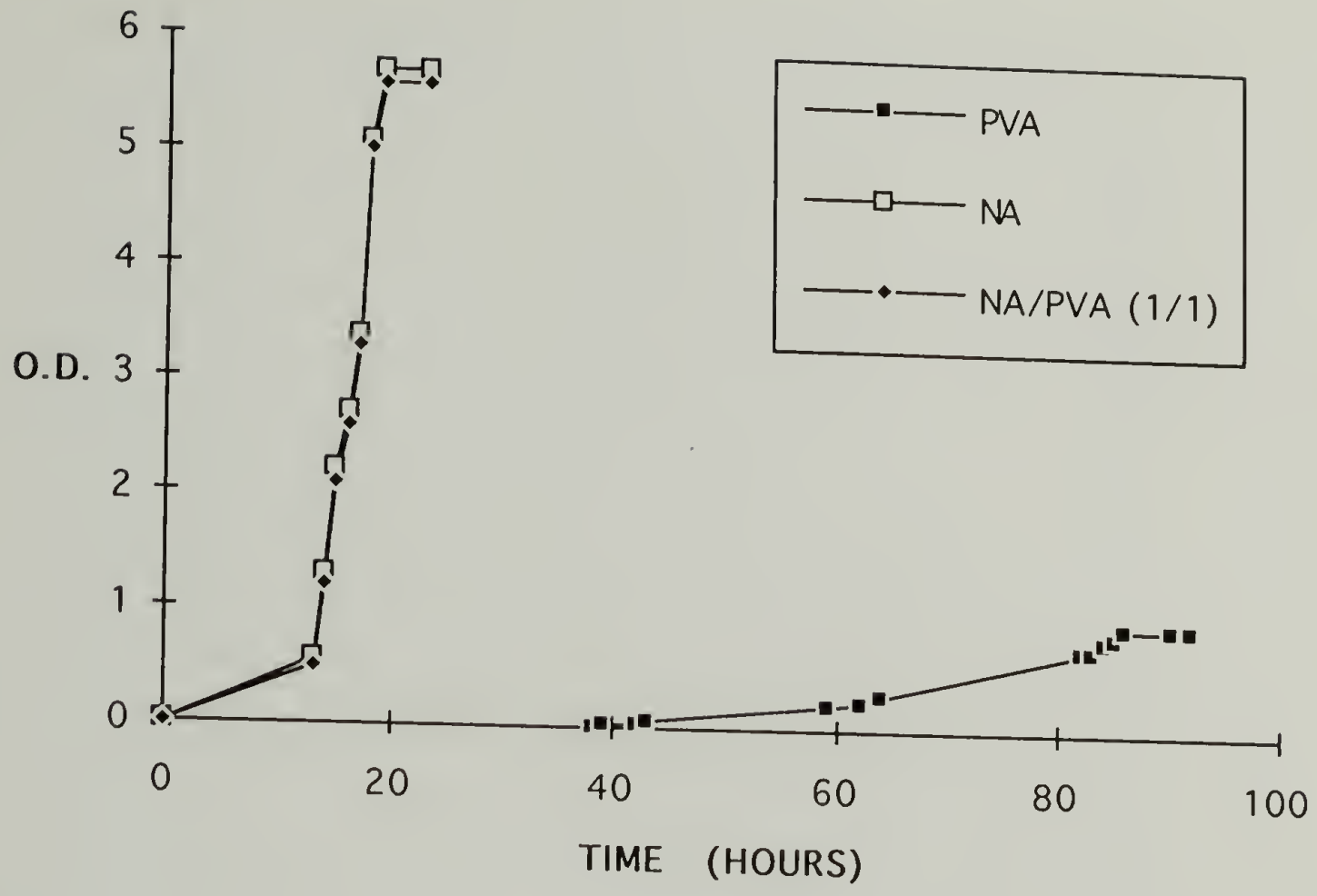


Figure 3.9 Growth curves of *P. oleovorans* grown on 5-phenylvaleric acid, PVA, nonanoic acid, NA, and an equimolar mixture of NA and PVA.

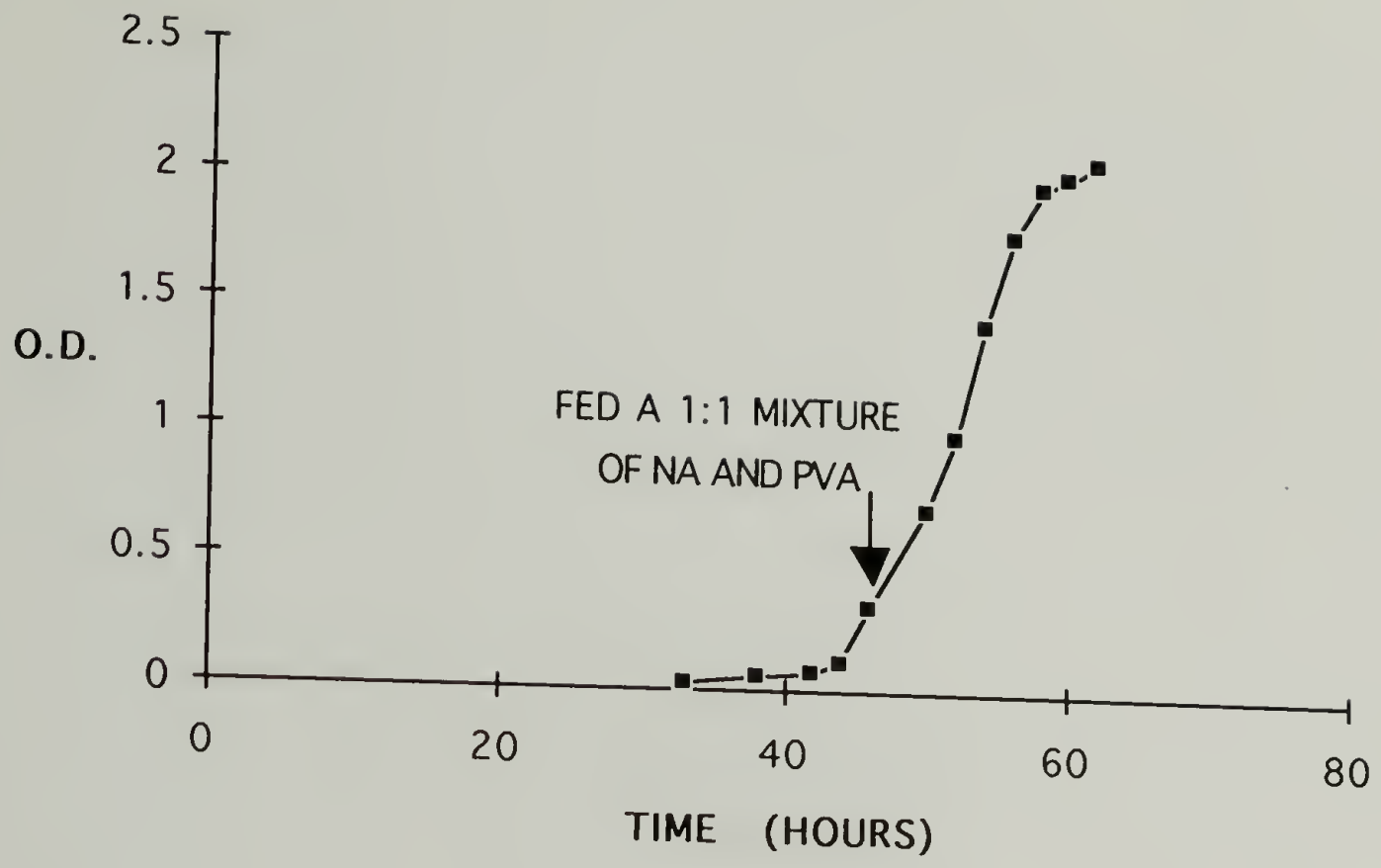


Figure 3.10 Growth curve obtained when *P. oleovorans* was initially grown on PVA as a sole carbon and then resuspended in an equimolar mixture of NA and PVA

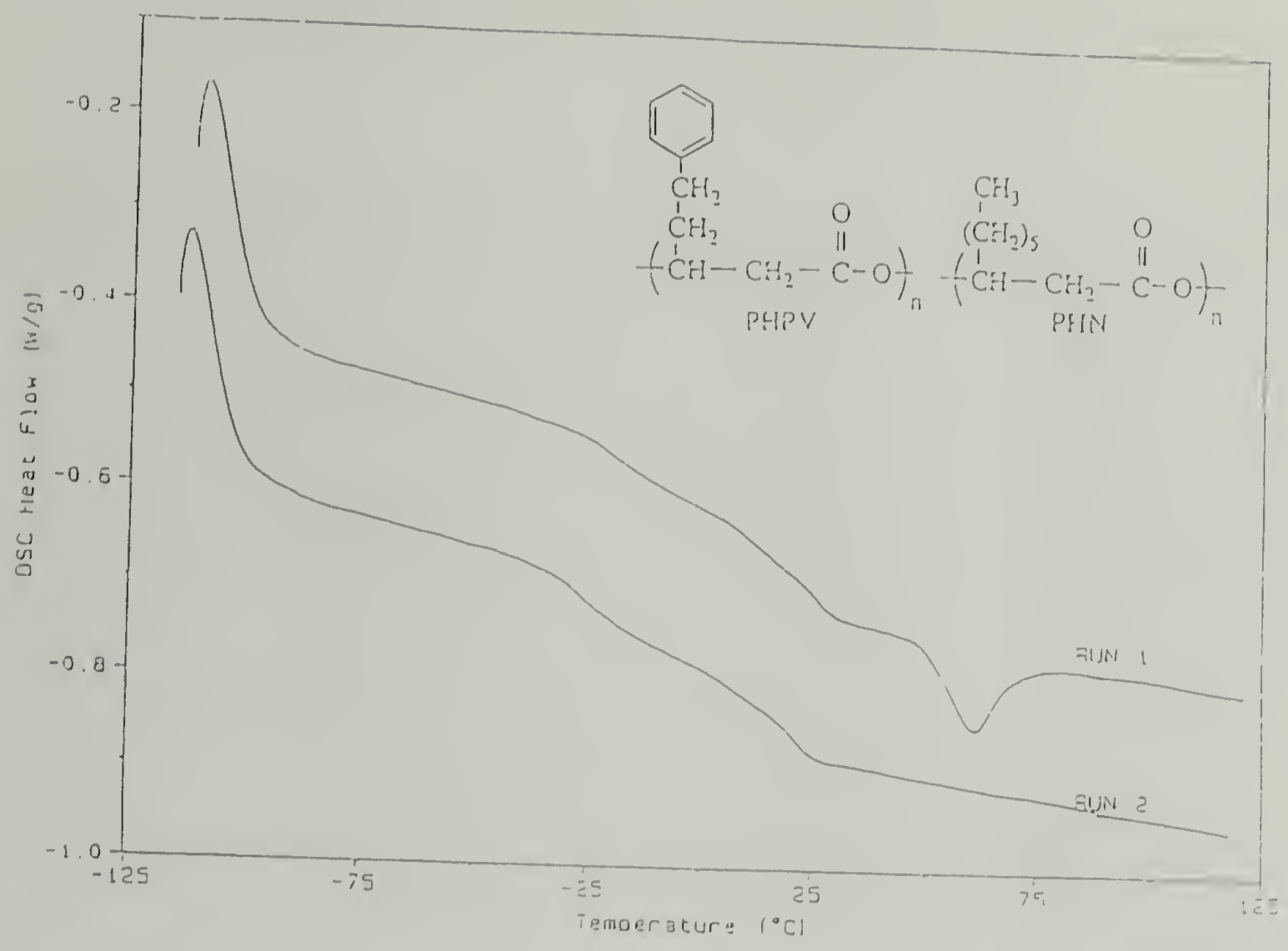


Fig 3.11 DSC thermograms of the polymer obtained when the bacteria was initially grown on PVA until the lag time was overcome, then centrifuged and resuspended in an equimolar mixture of NA/PVA.

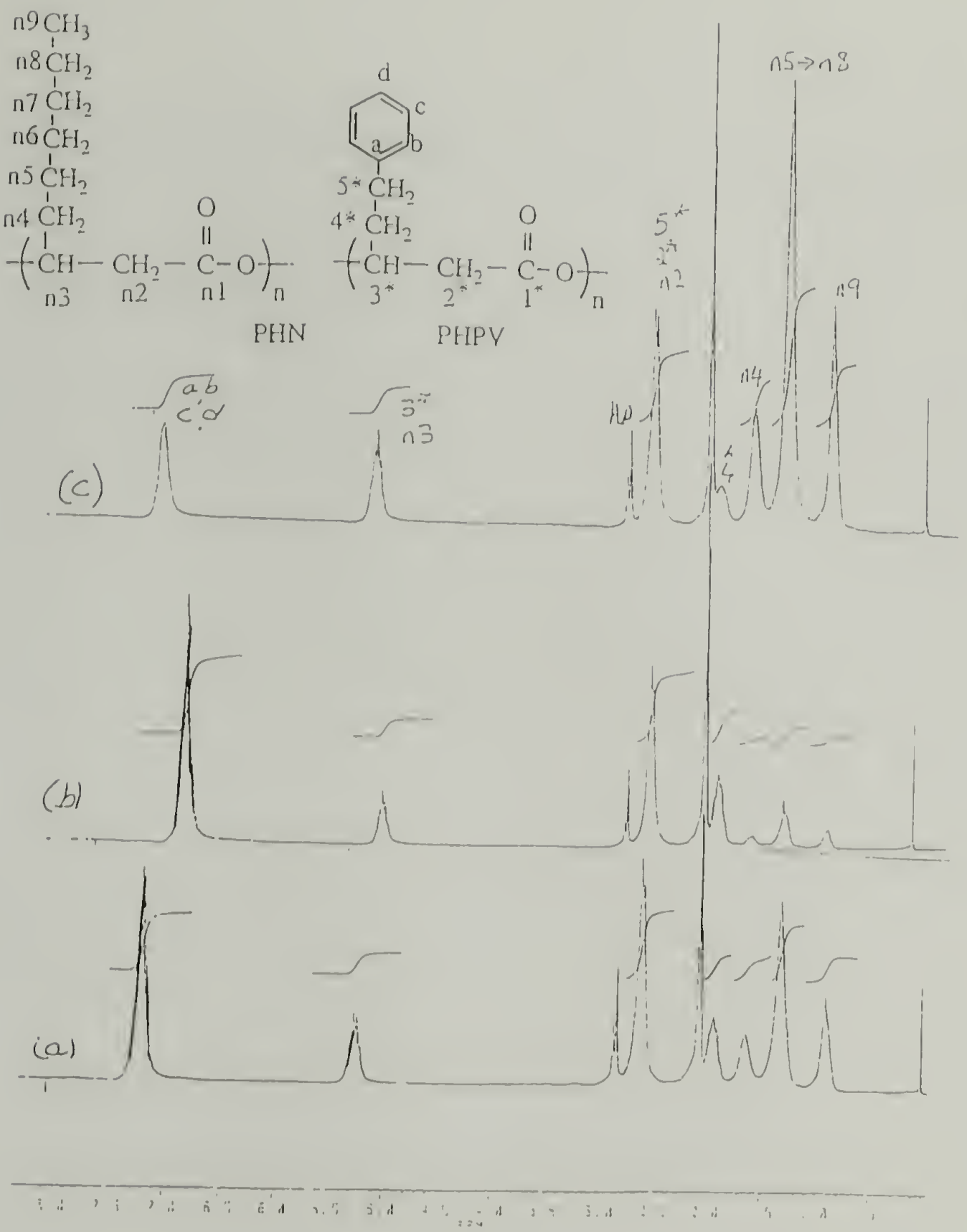


Fig 3.12 ^{1}H NMR of the polymer obtained when the bacteria was initially grown on PVA until the lag time was overcome, centrifuged and resuspended in an equimolar mixture of NA/PVA.(a) Polymer blend as precipitated from MeOH (52% phenylalkane repeating units), (b) Hexane insoluble polymer (84% phenylalkane repeating units), (c) Hexane soluble polymer (25% phenylalkane repeating units)

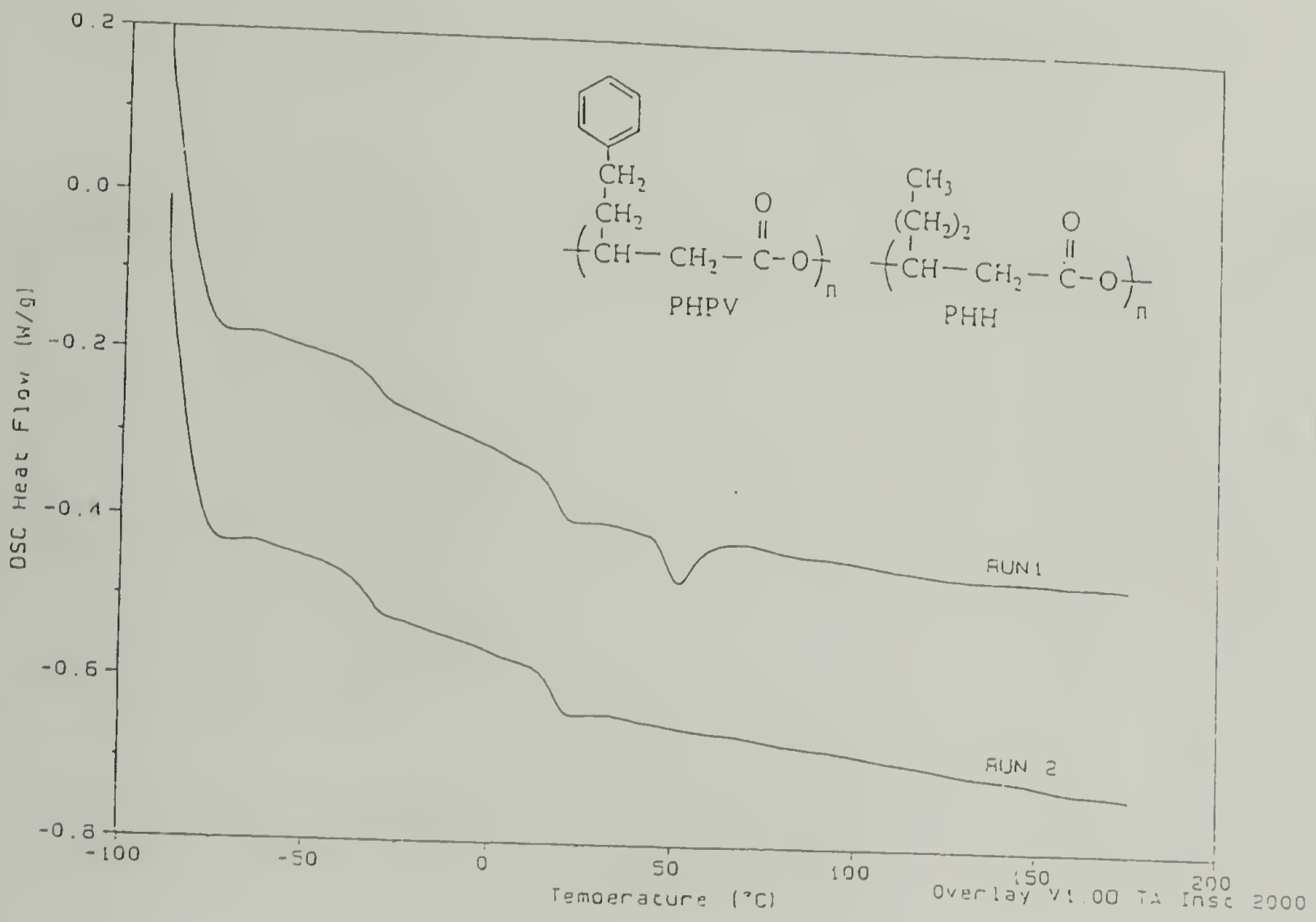


Fig. 3.13 DSC of the polymer obtained when the bacteria was grown on an equimolar mixture of hexanoic acid (HA) and 5-phenylvaleric acid (PVA)

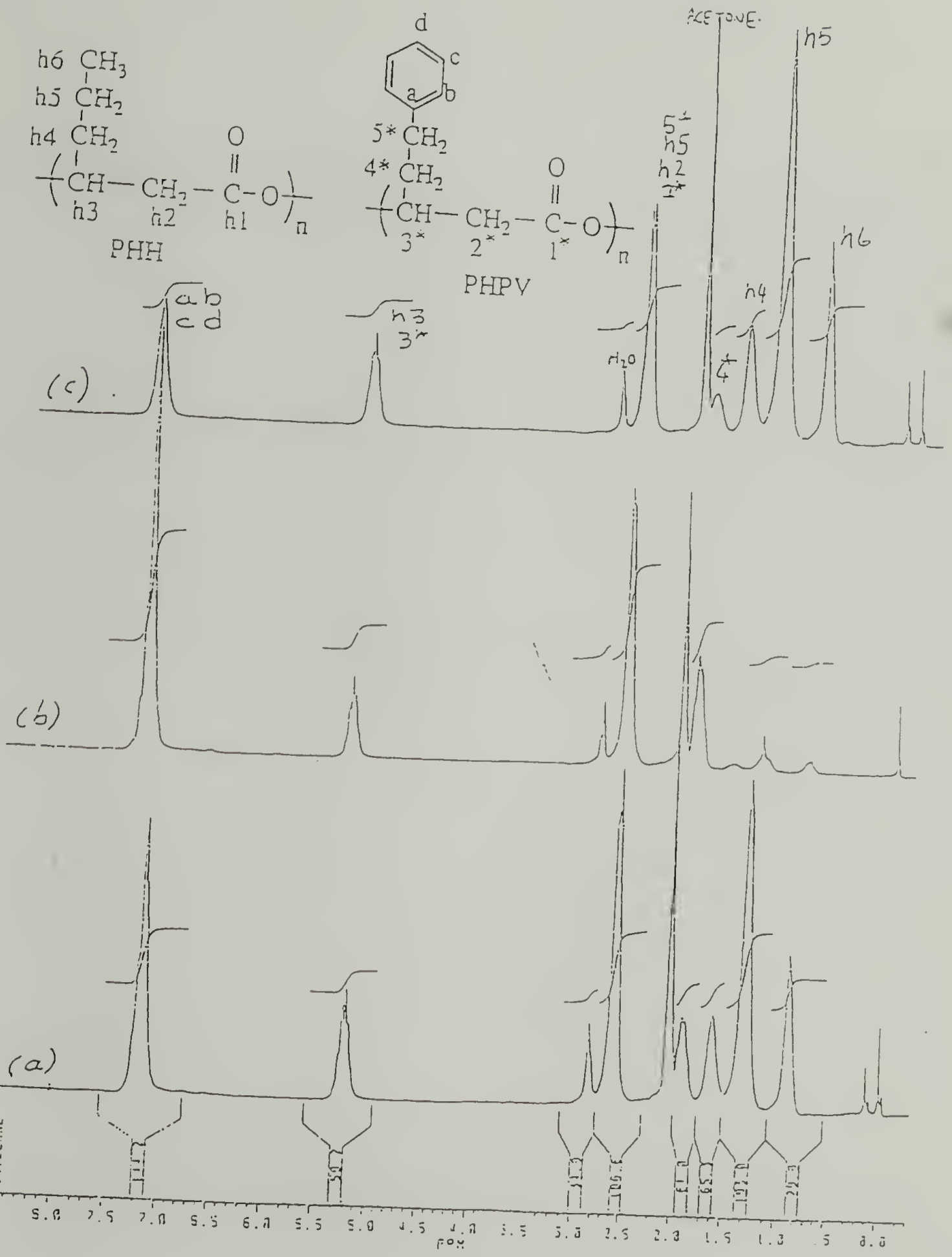


Fig. 3.14 ^{1}H NMR of the polymer obtained when the bacteria was grown on an equimolar mixture of hexanoic acid (HA) and 5-phenylvaleric acid (PVA) (a) Polymer blend as precipitated from MeOH (50% phenylalkane repeating units), (b) Hexane insoluble polymer (89% phenylalkane repeating units), (c) Hexane soluble polymer (29% phenylalkane repeating units)

Table 3.1. Growth condition and results obtained in the experiments to produce random copolymers

Substrates	Harvest Time hours	O.D. at Harvest	Cell Yield g/L	Polymer Yield g/L	% polymer % DW[a]
PVA then NA/PVA	25	3.4	1.3	0.2	14
HA/PVA	27	6.8	2.8	0.4	15

3.4 Conclusions

When *P. oleovorans* was grown on an equimolar mixture of NA and PVA, TEM results showed that a mixture of polymers were formed sequentially in the same granule with PHN synthesized in the core of the granule and PHPV formed around this core. The freeze fracture results were inconclusive due to the similarity in the freeze fracture patterns of PHN and PHPV.

SDS-PAGE results indicate that these two polymers were produced by the same enzyme system. The same polymerase enzyme bands were evident in SDS-PAGE when the bacteria was grown either on PVA to produce PHPV or on NA to produce PHN. No other bands, which may have been due to new or previously undetected polymerase enzymes, were observed when PVA was used as the sole carbon source. This result indicated that when the bacteria was cofed an equimolar mixture of NA and PVA, both PHN and PHPV were synthesized by the same enzyme system.

It was thought that, since both polymers were produced by the same polymer system, that it might be possible to manipulate the bacteria into producing a random copolymer with repeating units from both substrates, by changing the feed conditions and substrates. However, this objective was not realized. Characterization of the resulting

polymer revealed that it was a mixture of PHN and PHPV. It is concluded, that, while the same enzyme system is responsible for the production of both polymers, there is some distinction between the two substrates resulting in the production of two separate polymers.

3.5 References

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CHAPTER 4
INCREASING POLYMER YIELDS

4.1 Introduction

A constant challenge in the production of PHA's is increasing the polymer yield. As outlined in Chapter 1, PHA's can be divided into two categories, short-chain-length PHA's (SCL-PHA's) and medium-chain-length PHA's (MCL-PHA's). SCL-PHA's were discovered in the 1920's [1,2] whereas the first MCL-PHA was not discovered until 1974, when Wallen and coworkers extracted a copolymer which was composed of mainly 3-hydroxybutyrate and 3-hydroxyvalerate, but which also contained repeat units of 3-hydroxyhexanoate, 3-hydroxyheptanoate and 3-hydroxyoctanoate as minor repeat units. In general, the yields of SCL-PHA's are higher that those of MCL-PHA's. For example, *Alcaligenes eutrophus*, one of the bacteria most commonly used to produce SCL-PHA, has produced a cell yield of 8.3g/L and 90% polymer based on dry cell weight [3].

Pseudomonas oleovorans, Pseudomonas putida, Pseudomonas citronellolis , and *Pseudomonas aeruginosa* are examples of PHA-MCL-producing bacteria. Cell yields of approximately 7g/L, with a polymer yield of 35% based on dry cell weight were obtained using *P. oleovorans* [4].

There are a number of reports on the different types of fermentations used to produce PHA's. Zeneca Ltd. uses a two-stage fermentation process in its production of "Biopol" [5]. In the first stage the bacteria are grown on an inexpensive abundant carbon source, glucose, until the supply of phosphate is limited. Then the carbon source, which will produce the PHA, is added to this nutrient-deficient medium. In a variation of this method, which is possible for smaller scale production, the culture is centrifuged at the onset of the limitation of an essential nutrient, and the cells are resuspended in a nutrient-deficient medium, which has an abundance of the carbon source that is used to produce the polymer [6,7].

In an earlier study in this laboratory, standard conditions which were developed for a 12L fermentor included a stirring rate of 100 rpm, an air flow rate of 2L/min and a carbon substrate concentration of 20mM, which was added to the culture prior to autoclaving [8] With *P. oleovorans,* these conditions produced approximately 1g/L of dry cell material and 25% polymer based on cellular dry weight. More recently in this laboratory, Gagnon [4] showed that by increasing the cell density, the polymer yield based on cellular dry weight could be increased. The cell density was increased by using an air flow of 6ml/min and feeding 10mM of carbon source to the culture, either periodically or whenever the O.D. increased by 2 units. The periodic feeding of the carbon source was performed either manually or by using a peristaltic pump attached to a timer. Using these conditions, 7g/L of cell material containing up to 35% polymer (based on cell dry weight) was obtained.

The goal of this section of the dissertation research program was to increase the yields of MCL-PHA's, in particular, the yield of poly-3-hydroxyphenylvalerate (PHPV), and to decrease polymer production costs. Previous studies have shown that the usual amount of polymer that *P. oleovorans* can store is 25-35% of the cell dry weight [4]. Therefore, in order to increase polymer yields it was necessary to increase the amount of cells formed. Four strategies were undertaken to increase the number of cells produced in the culture. The first three strategies used the same bacteria *Pseudomonas oleovorans,* and the fourth strategy used a strain of *Pseudomonas putida*.

The first strategy was to feed the carboxylic acid to the bacteria a number of times, multiple feeding, to increase the number of cells produced. The second strategy involved prefeeding the bacteria a less expensive carbon substrate which was used by the bacteria in its routine metabolic cycle. Then, when the bacteria had reached the stage where one of its essential nutrients was limited, PVA was added to the culture. Addition of PVA at the appropriate time ensured that this expensive substrate would mainly be used in polymer production. The third strategy was to initially grow the bacteria on a 'rich' medium. When the stationary growth phase was attained the culture was centrifuged and resuspended in E*

minimal media, which contained various carboxylic acids as the carbon source. The idea was that polymer accumulation should take place in cells placed in this minimal medium. This technique has been used very successfully by Doi and coworkers to produce copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate [9] and also to produce copolymers of 3-hydroxybutyrate, 3-hydroxyvalerate and 5-hydroxyvalerate [7] using *Alcaligenes eutrophus*.

The fourth strategy undertaken was based upon a recent report from Yoon and coworkers [10] in which a strain of *Pseudomonas putida* which had been isolated from soil in a landfill, was found to incorporate a higher proportion of phenyl moieties than other *Pseudomonads* when grown on a mixture of NA and PVA. A sample of that bacterium, which has been labeled *P. putida* BM01 was kindly donated to our research group by Yoon, so that the polymer production from *P. putida* could be compared with that from *P. oleovorans*.

As the goal of this portion of the dissertation research program was to increase the yields of MCL-PHA's, optical microscopy was used to screen cultures for intracellular inclusion bodies. The cultures were stained with Nile Blue A, a stain which causes PHB granules to exhibit a strong orange fluorescence when exposed to light of 460nm [11]. The flexibility of this technique for detecting other types of PHA granules was also tested in this study.

4.2 Experimental

4.2.1 Nile Blue Staining for Optical Microscopy

A thin layer of the bacterial culture to be examined was placed on a glass slide. The sample on the slide was fixed by passing it over a flame. The slide was allowed to dry and was then exposed to a 1% Nile Blue A stain solution in a Coplin jar at 55°C for 10 minutes. Excess dye was removed by a water wash and the slide was placed in a Coplin jar containing an 8% acetic acid solution for 1 minute. The stained bacterial smear was blotted

dry with bibulous paper, remoistened with water, and covered with a coverslide. The water created a barrier between the cell and the lens oil because the oil could dissolve and remove the stain. The slide was viewed using a phase contrast optical microscope.

4.2.2 Production of PHA's by *P. oleovorans*

4.2.2.1 Multiple Feeding

The cultures were grown in a 12L New Brunswick fermentor using either NA or PVA as the sole carbon source. The dissolved oxygen was measured using an oxygen probe and a chart recorder. A stirring rate of 200rpm, an air flow of 5L/min., and a temperature of 30°C was used. Multiple feeding was performed by either adding the substrate manually, whenever the dissolved oxygen (D.O.) increased, or by automatically feeding at preset times. The automatic feeding system consisted of a peristaltic pump and a programmable timer, which were used to add substrate to the culture at preset times.

4.2.2.2 Prefeeding Experiments

The bacteria were initially grown on an inexpensive substrate, then, at the onset of the polymer production, PVA was added to the culture. It should be noted that while *P. oleovorans* grows on substrates of carbon chain length from one to sixteen carbons, it only produces polymer on those of carbon chain length between six and sixteen carbons [12]. . Four 'prefeeding' substrates which were used were as follows:
(1) sodium acetate, an inexpensive substrate on which the cells grow but do not produce polymer (a "poorer" substrate); (2) sodium butyrate, also a substrate on which the cells grow but do not produce polymer; (3) nonanoic acid, NA, on which the bacteria grow readily and produce polymer (a "good" substrate) and (4) valeric acid, another "poor" substrate on which the bacteria grow. but do not produce polymer.

These bacterial cultures were grown as outlined in Section 2.2 using one of the above prefeeding substrates. At the onset of the stationary growth phase, either the cultures

were centrifuged and resuspended in medium which contained PVA as the sole carbon source or PVA was added to the culture. Aliquots from the cultures were harvested periodically to determine the optimum harvest time to achieve the maximum polymer yield.

4.2.2.3 Rich Media Experiments

P. oleovorans was initially grown on a "rich medium" which consisted of the following: 5g/L trypticase peptone, 5g/L peptone, 10g/L yeast extract, 5g/L meat extract, 5g/l $(NH_4)_2SO_4$. The pH was adjusted to 7. This medium was inoculated from a plate of *P. oleovorans* which had been stored at 4^0C. When the stationary growth phase was achieved the culture was centrifuged at 4000 rpm for 20 minutes and resuspended in E* minimal media containing PVA as the sole carbon source. Two types of E* minimal media were used, one which contained 1.1g/L of $(NH_4)_2HPO_4$ and one which contained no $(NH_4)_2HPO_4$. No further increase in O.D. was observed upon resuspension of the cells in E* medium, and the culture was harvested 24 hours later.

4.2.3 Production of PHA's by *P. putida*

P. putida BM01 was grown in a mineral media termed the "Yoon Media" which consisted of the following: 1.06g/L $(NH_4)_2SO_4$, 2.129 g/L $NaHPO_4$, 2.0141g/L KH_2PO_4, 10ml/L of a 100mM solution of $MgSO_4$, and 1ml/L of a microelement solution. The microelement solution consisted of the following in 1M HCL: 2.78g/L $FeSO_4.7H_2O$, 1.67g/L $CaCl_2.2H_2O$, 0.17g/L $CuCl_2.2H_2O$, 0.29g/L $ZnSO_4.7H_2O$, 1.98g/L $MnCl_2.4H_2O$, 2.81g/L $CoSO_4.7H_2O$. The cultures were grown as outline in Section 2.2.

4.3 Results and Discussion

4.3.1 Nile Blue Staining of PHPV

Nile Blue A has previously been used to stain PHB [11]. Figure 4.1a and b show the effect that exposure to light of 460nm had on the Nile Blue A-stained granules of

PHPV. Figure 4.1a is an optical micrograph of *P. oleovorans* containing PHPV intracellular granules, and Figure 4.1b shows the same field of view when these PHPV granules are exposed to an excitation wavelength of 460nm, which caused the PHPV to fluoresce bright orange. From these photographs it can be seen that this staining technique is not restricted to PHB and can be a valuable tool for screening bacteria for PHA inclusion bodies which contain MCL-PHA's and even functional polymers

4.3.2 Production of PHA's by *P. oleovorans*

4.3.2.1 Multiple feeding Experiments

The growth curves for the multiple feeding with NA and PVA are shown in Figure 4.2a and b. Multiple feeding of *P. oleovorans* with NA resulted in a final O.D. of 12 and a polymer yield of 1g/L, compared to an O.D. of 3 and a polymer yield of 0.25g/L when the bacteria were fed once. The results obtained from the multiple feeding of PVA were not as dramatic. The final O.D. increased from 1.5 to 1.9, and the polymer yield increased from 0.1g/L to 0.12 g/L.

4.3.2.2 Prefeeding Experiments

Seven 1L cultures were prepared, 5 of which contained 50mM of sodium acetate, and the other two contained 50mM of sodium butyrate as a pre-feeding source. The cultures grew to an optical density of approximately 1.0 before the PVA was added, and were harvested between 14 and 172 hours later. The harvest time, optical density, cell yield, polymer yield and % polymer (based on the cell dry weight) are given in Table 4.1. The use of sodium acetate and sodium butyrate as prefeeding sources resulted in lower yields of PHPV polymer than if *P. oleovorans* had been grown on PVA from the start of the experiment.

Table 4.2 contains the results obtained when the bacteria was grown on NA prior to the addition PVA. The amount of polymer obtained was higher (15-20%) when NA was

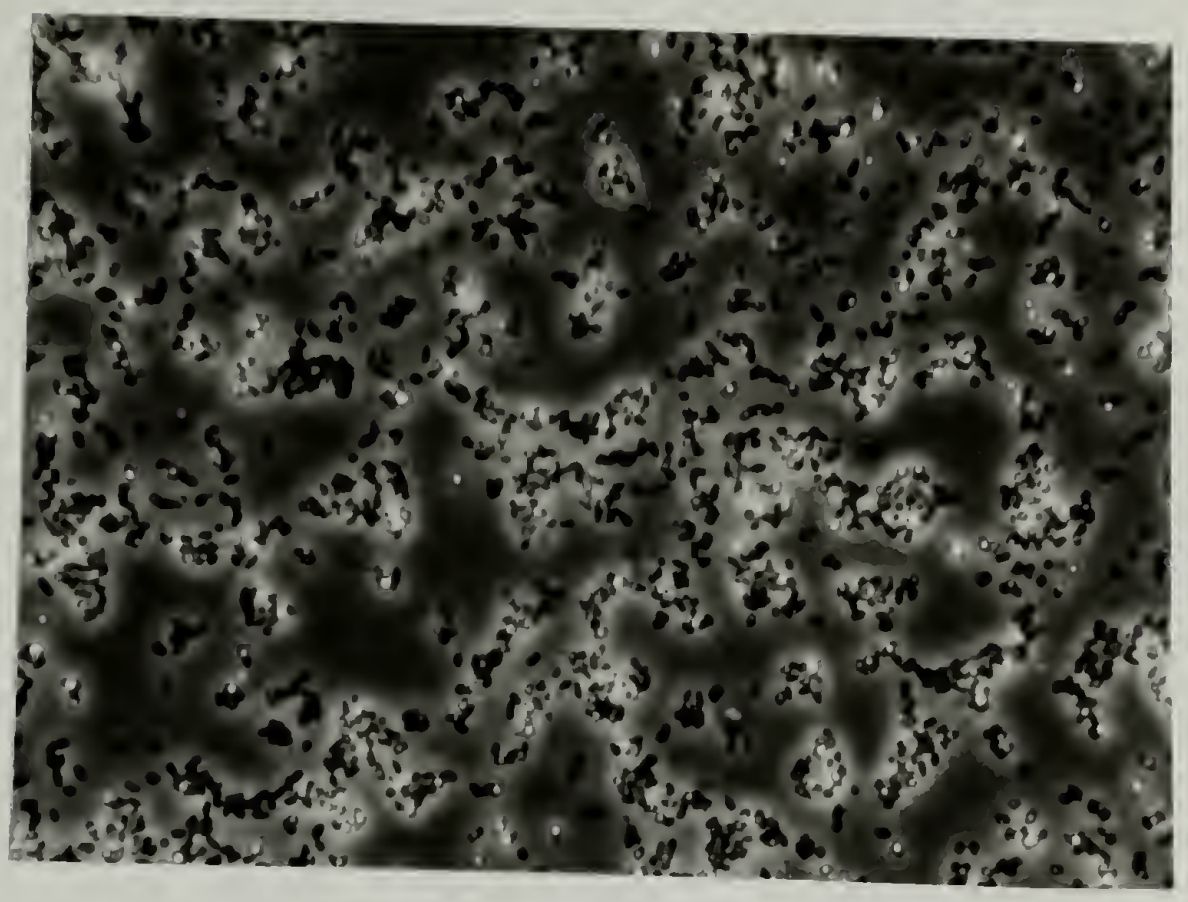
used as the prefeeding source than when sodium acetate or sodium butyrate were used as the prefeeding sources (5-11%). However, when NA was used most of the polymer formed was PHN whereas when sodium acetate and sodium butyrate were used as prefeeding substrates 100% of the polymer produced was PHPV. It appears, therefore, that *P. oleovorans* did not adapt well to changes in feed substrate.

Valeric acid was also used as a prefeeding substrate. As is shown in Figure 4.3, *P. oleovorans* was grown on valeric acid until a maximum O.D. was achieved, after which PVA was added to initiate polymer production. The objective in this case was to induce the bacteria into oxidizing valeric acid, so that they might react favorably to PVA and produce high yields of PHPV. The cell yield was 0.61 g and the amount of polymer based on cellular dry weight was 5.9%. Although the time taken to produce PHPV in this case was shorter than when PVA was the sole carbon source, this experiment did not generate a high enough yield to be considered as an improved method for PHPV production.

4.3.2.3 Rich Media Experiments

Because none of the prefeeding experiments resulted in an increase in the yield of PHPV polymer, the next experiment carried out was one in which *P. oleovorans* was initially grown on rich media until a maximum O.D. of 6 was achieved, then the culture was centrifuged and resuspended in E* media containing either NA or PVA as the sole carbon source. The growth curves for this experiment are shown in Figure 4.4. There was no increase in O.D. upon resuspension of the cells in E* media, and the polymer yields were less than when the bacteria was grown on E* media from the start. In addition, the elimination of nitrogen from the E* media did not result in an increase in the amount of polymer incorporated. This result indicates that while the cells grew well on rich media, *P. oleovorans* did not react well to being transferred from one type of media to another.

(a)



(b)

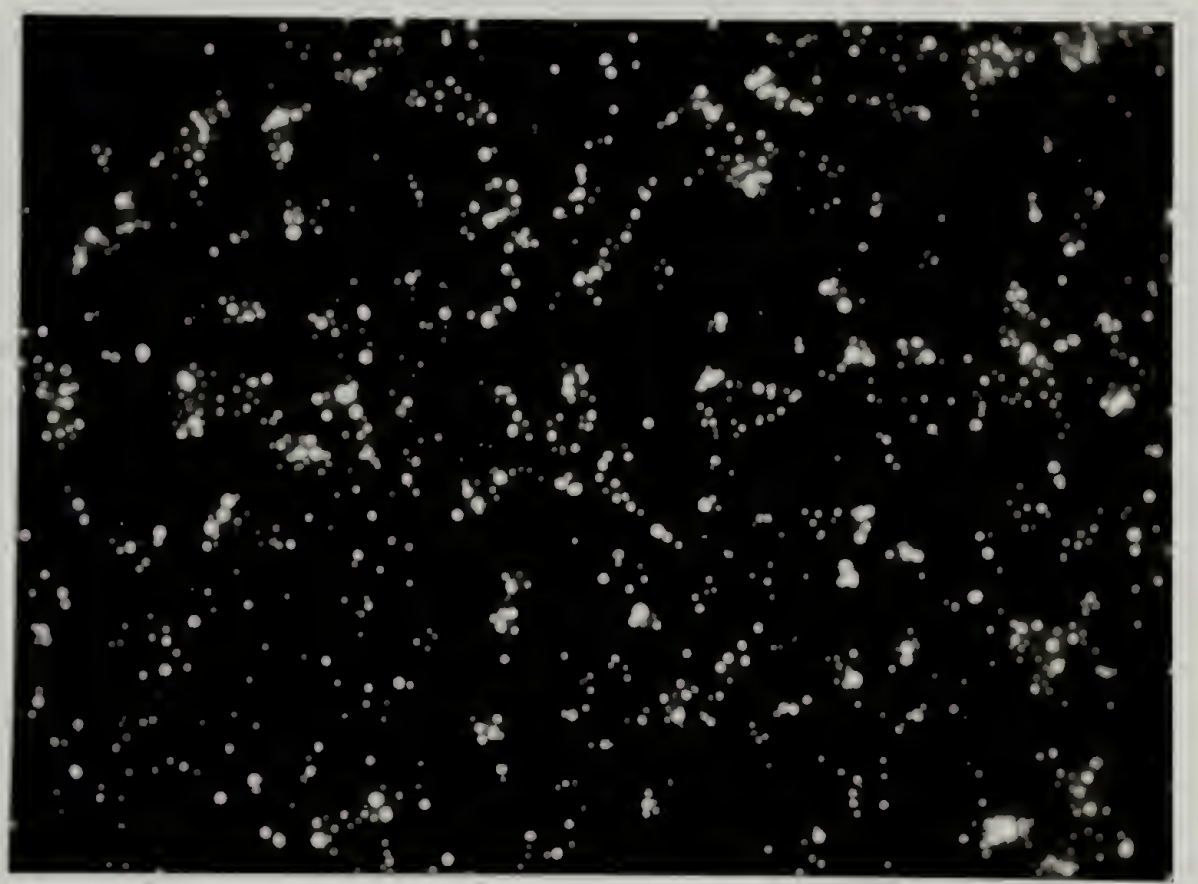
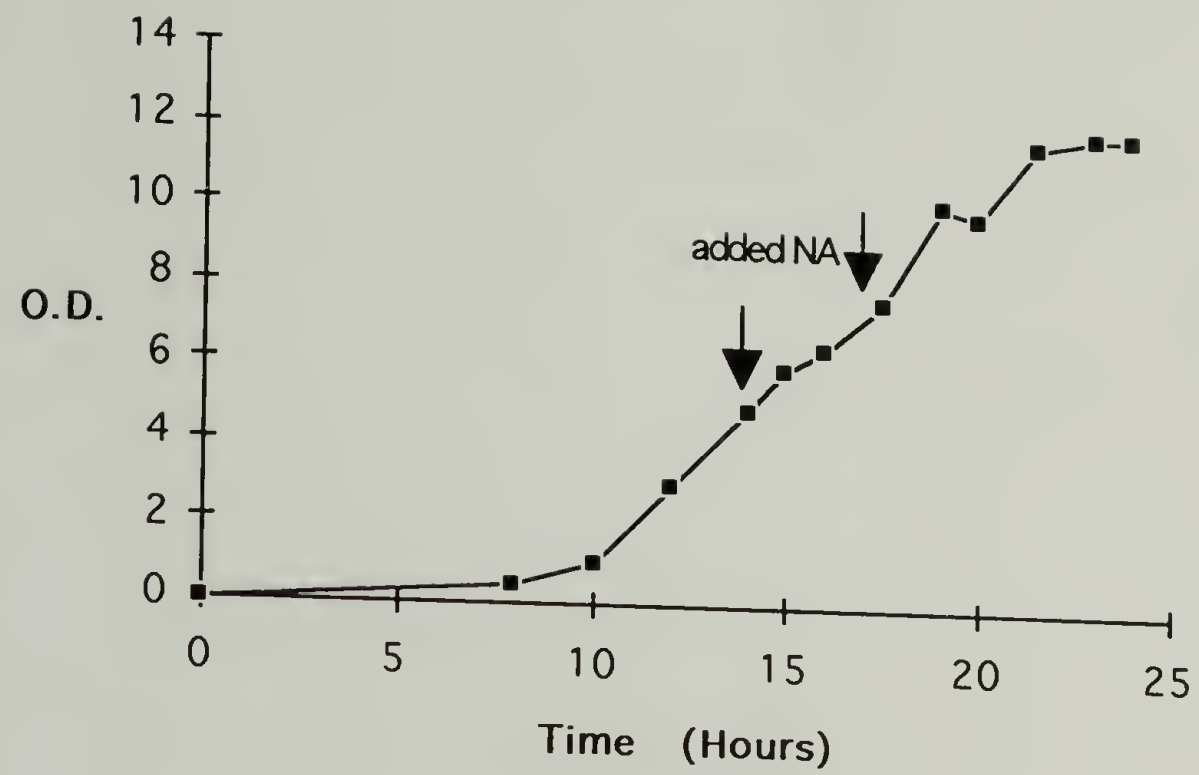


Figure 4.1(a) Photomicrograph of *P. oleovorans* containing PHPV intracellular granules (b) Photomicrograph of the same field of view as 4.1a, when the PHPV granules were exposed to an excitation wavelength of 460nm, causing the PHPV to fluoresce

(a)



(b)

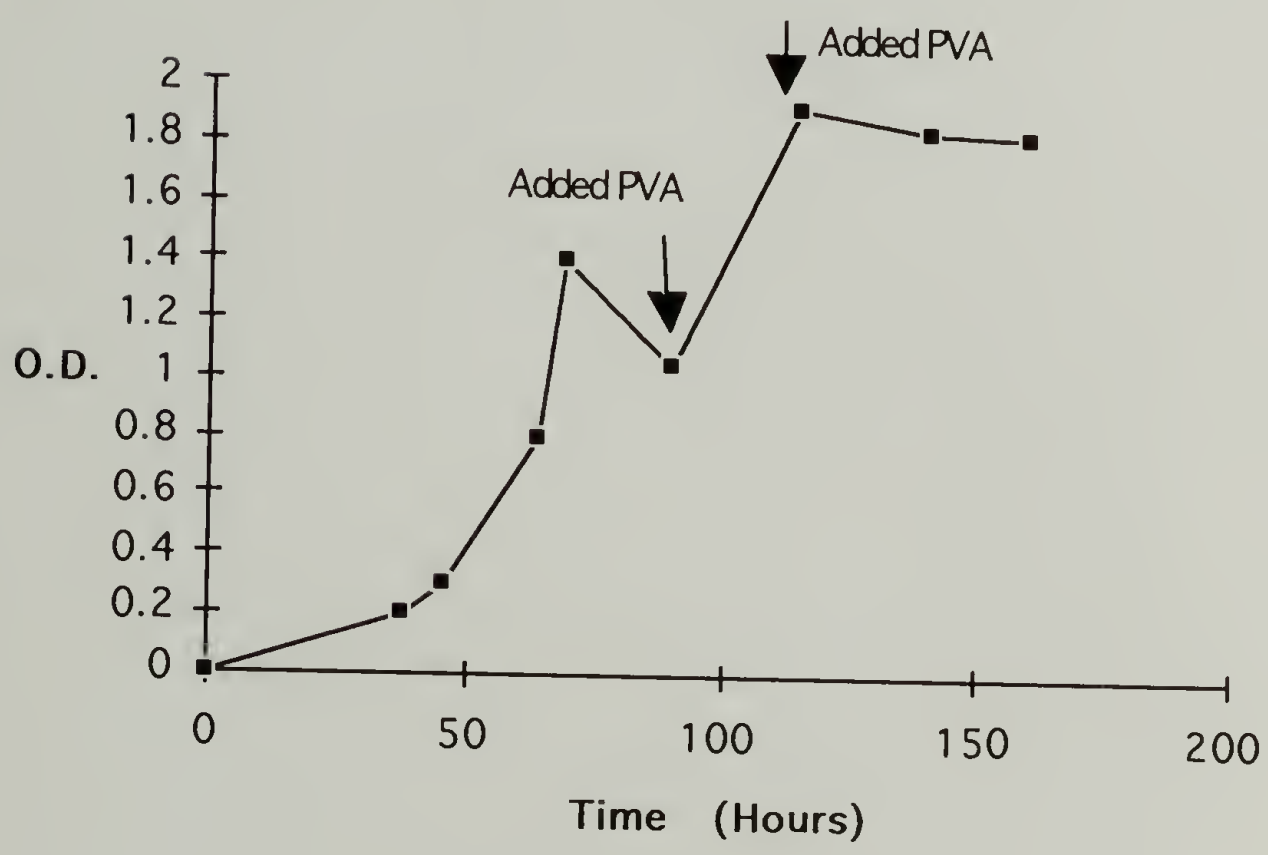


Figure 4.2 Growth curve of *P. oleovorans* grown on (a) NA and (b) PVA using the multiple feeding method

Table 4.1 Growth conditions and results obtained when *P. oleovorans* was grown on sodium acetate or sodium butyrate prior to the addition of PVA.

Prefeeding Substrate	Harvest Time, hours	O.D. at Harvest	Cell Yield, g/L	Polymer Yield, g/L	% Polymer %DW a
Na acetate	14	2.3	1.6	.08	5.3
Na acetate	45	2.3	0.7	.03	4.3
Na acetate	70	2.3	0.7	.05	7.4
Na acetate	109	2.5	0.7	.03	4.7
Na acetate	172	0.9	0.3	.03	11.3
Na butyrate	48	1.9	1.1	.025	2.6
Na butyrate	72	2.0	1.1	.03	3.1

a: Polymer yield based on cell dry weight

Table 4.2 Growth conditions and results when *P. oleovorans* was grown on NA prior to the addition of PVA.

Harvest Time, hours	O.D. at Harvest	Cell Yield, g/L	Polymer Yield, g/L	Polymer Yield, % DW[a]	% Phenyl-Containing Repeating Units
14	3.4	2.3	0.53	23	0
24	2.7	1.9	0.39	20	0
70	8.6	3.2	0.47	15	6.7
172	4.6	2.0	0.39	20	0
228	7.3	2.6	0.42	16	32

a: Polymer yield based on cell dry weight

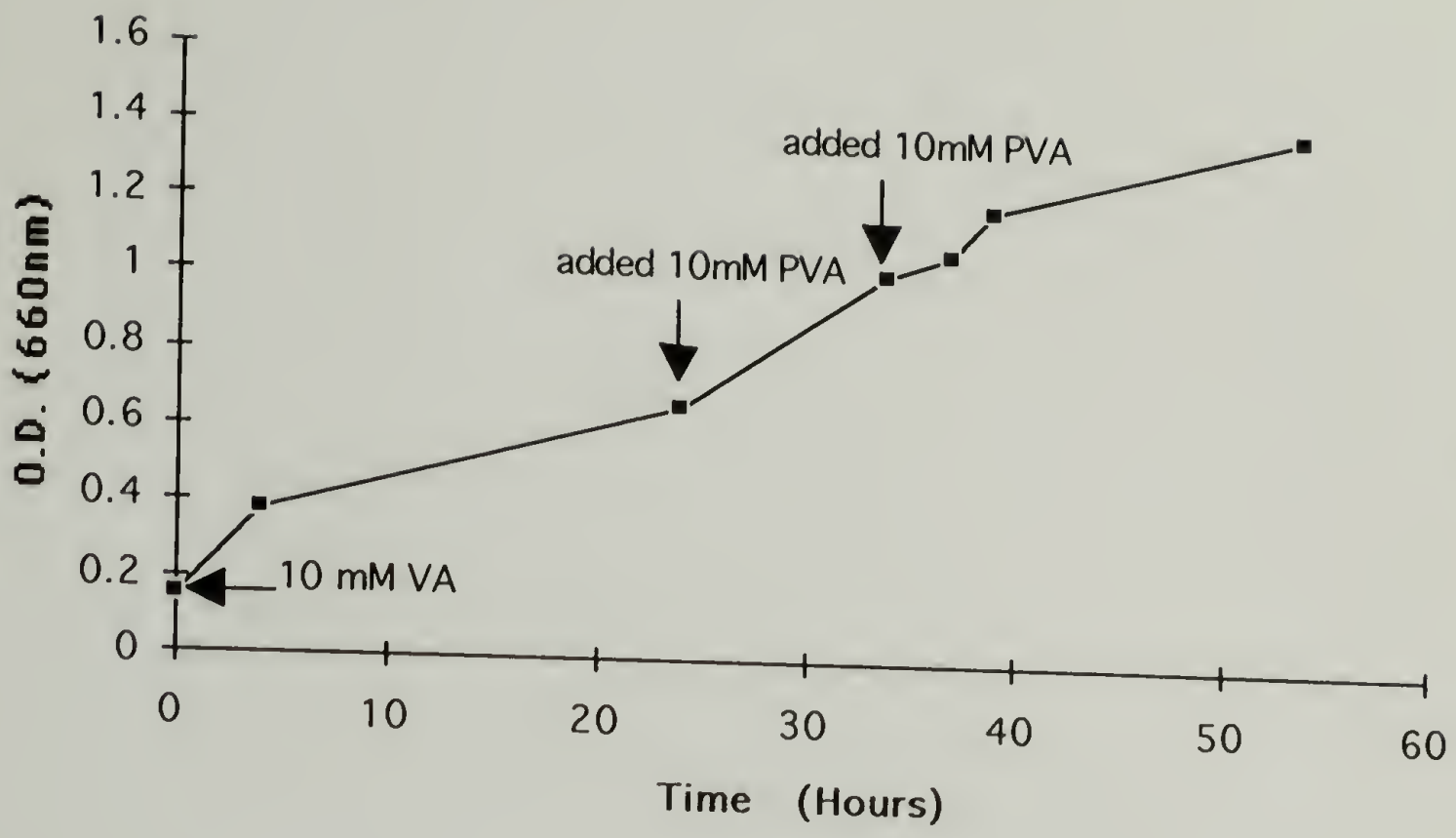


Figure 4 3 Growth curve obtained when *P. oleovorans* was initially grown on valeric acid prior to the addition of PVA

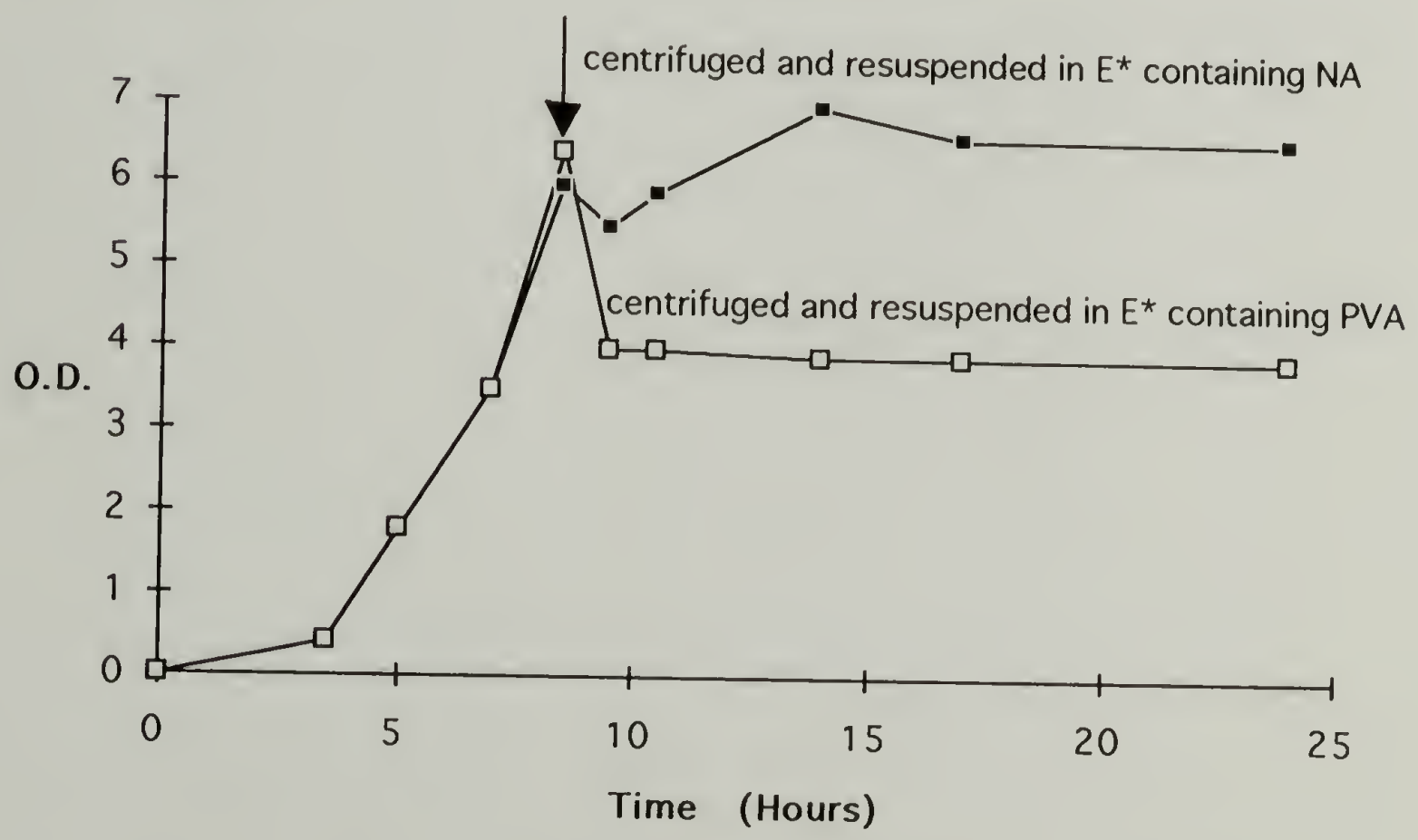


Figure 4.4 Growth curves obtained when *P. oleovorans* was grown on rich media and then transferred to E* media containing either NA or PVA as the sole carbon source.

4.3.3 Production of PHA's by *P. putida*

A recent report from Yoon and coworkers [10] stated that *P. putida* was more efficient than other *Pseudomonads* at incorporating phenyl-containing repeating units into the PHA backbone. As the goal of this section of the dissertation research program was to produce a high yield of PHPV as economically as possible, *P. putida* was investigated because this microorganism might prove more efficient than *P. oleovorans* at producing high yields of phenyl-containing polymer.

The first experiments carried out were to optimize the media and substrate concentration when *P. putida* was used. NA was used as the sole carbon source and both Yoon media and E* media were used. The growth curves obtained in this experiment are shown in Figure 4.5, and the cell yields, and polymer yields are given in Table 4.3. The maximum cell yield and PHN yield were obtained when *P. putida* was grown on 40mM of NA in Yoon media.

Figure 4.6 contains the growth curves obtained when *P. putida* was grown on NA, PVA and an equimolar mixture of NA/PVA. Figure 4.7 contains the growth curves obtained when *P. oleovorans* was grown on NA, PVA and an equimolar mixture of NA/PVA. From Figure 4.6 and 4.7 it can been seen that *P. putida* exhibited better growth than *P. oleovorans* when both bacteria were grown on PVA, and also, the cell yields and polymer yields were higher. *P. putida produced* 0.90g/L of cells and 0.16g/L of polymer whereas *P. oleovorans* produced 0.50g/l of cells and 0.11g/L of polymer.

Various different ratios of PVA:NA [1:1, 3:1] were fed to both *P. oleovorans and P. putida* to compare cell yields, polymer yields and the amount of phenyl-containing repeating units produced. The resulting growth curves are shown in Figure 4.8 and the maximum O.D., cell yield, polymer yield and the % phenyl-containing polymer obtained using each bacteria are tabulated in Table 4.3. In all cases, *P. putida* exhibited better growth than *P. oleovorans* when both bacteria were grown on the same carbon source.

When an equimolar mixture of NA and PVA was fed to each baeteria, 0.96g/L of polymer containing 23% phenyl repeating units were obtained using *P. putida* where as 0.37g/L of polymer containing 45% phenyl repeat units was obtained using *P. oleovorans* . When the ratio of PVA to NA was 3:1, *P. putida* produced 0.24g/L of polymer which eontained 79% phenyl repeat units and *P. oleovorans* produced 0.10g/L of polymer which contained 86% phenyl repeat units .

In contrast to the results of Yoon and coworkers [10], this study did not find that *P. putida* produced polyesters with a higher mole % phenyl repeating units in the polymer backbone, when eompared to the polymer produeed by *P. oleovorans* when grown on the same earbon sources. However, *P. putida* proved to be superior to *P. oleovorans* in that it produced a higher O.D., higher eell yield, and a higher polymer yield regardless of the substrate on whieh it was grown.

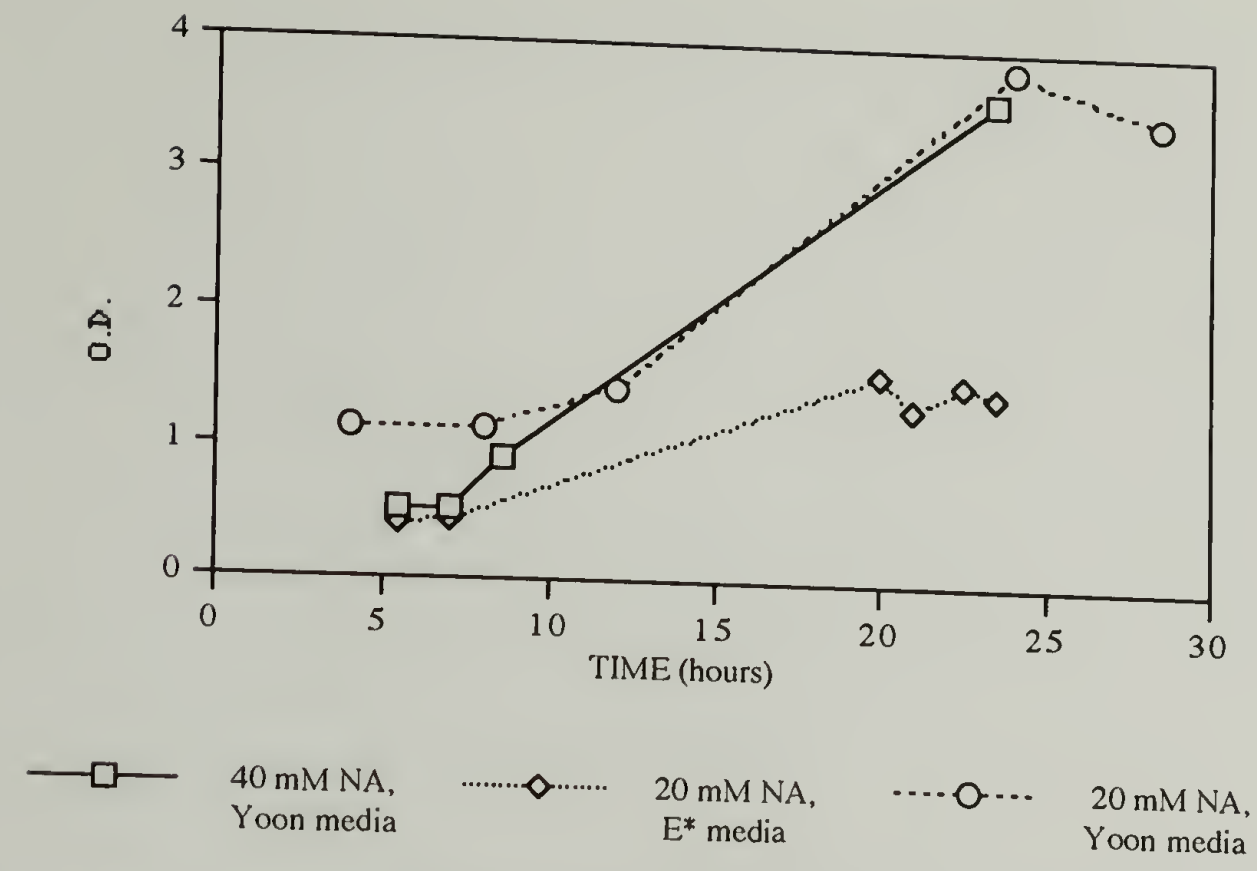


Figure 4.5 Growth curves obtained when *P. putida* was grown on different concentrations of NA in different media

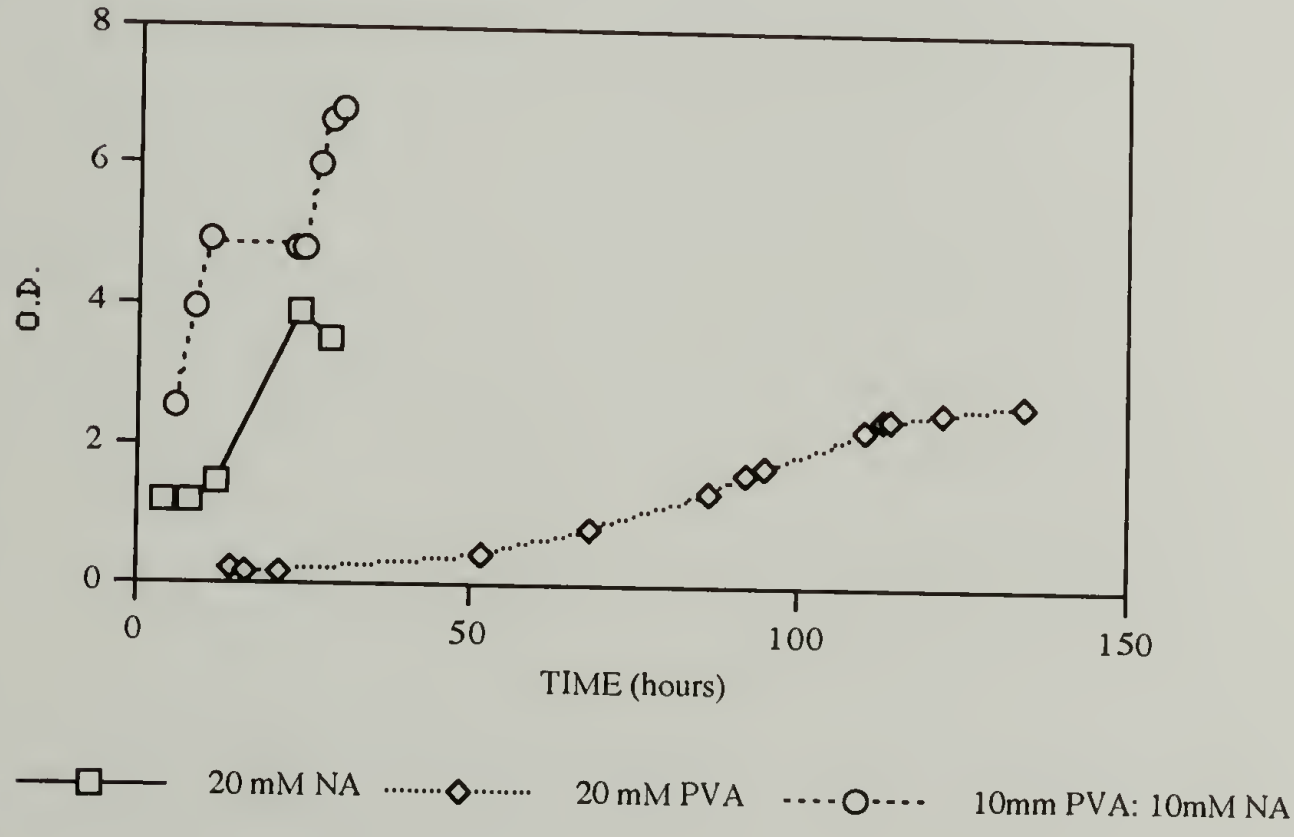


Figure 4.6. Growth curves obtained when *P. putida* was grown on NA, PVA and an equimolar mixture of NA and PVA

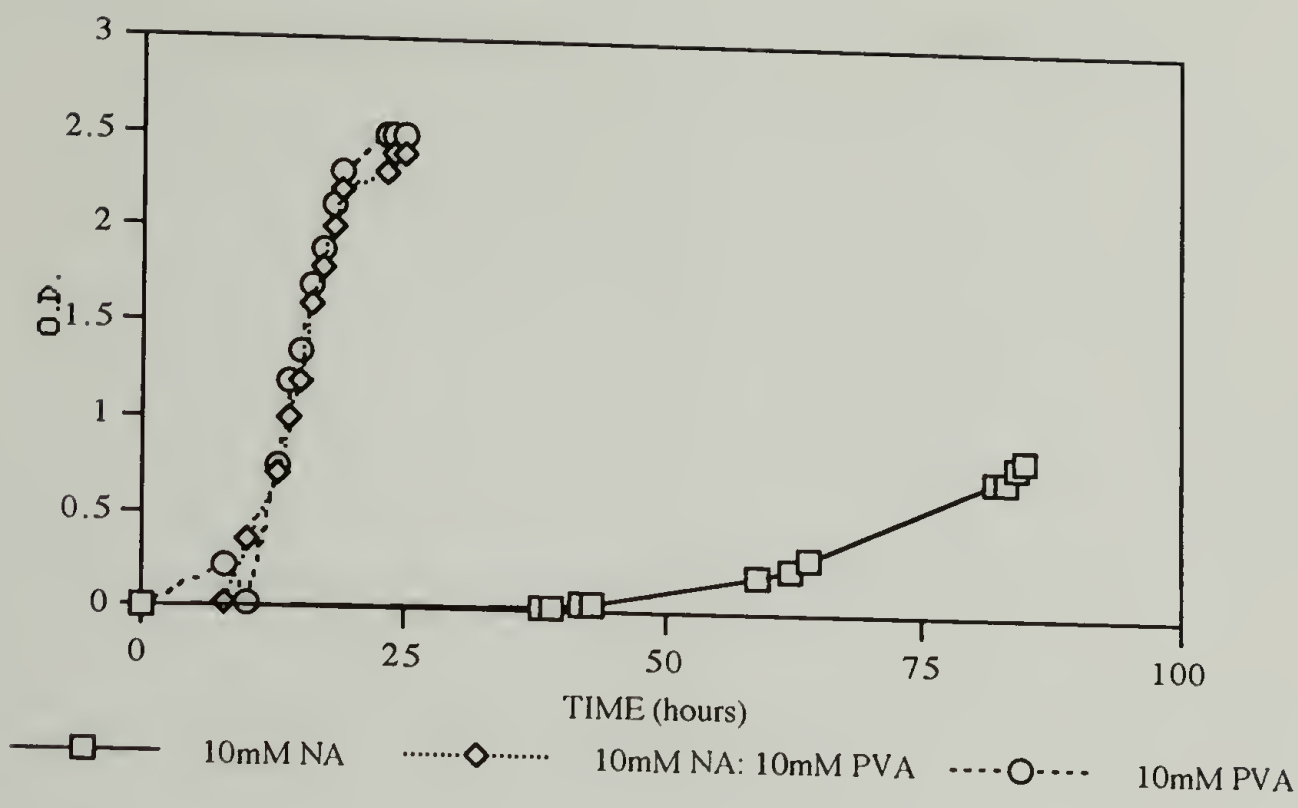


Figure 4.7. Growth curves obtained when *P. oleovorans* was grown on NA, PVA and an equimolar mixture of NA and PVA

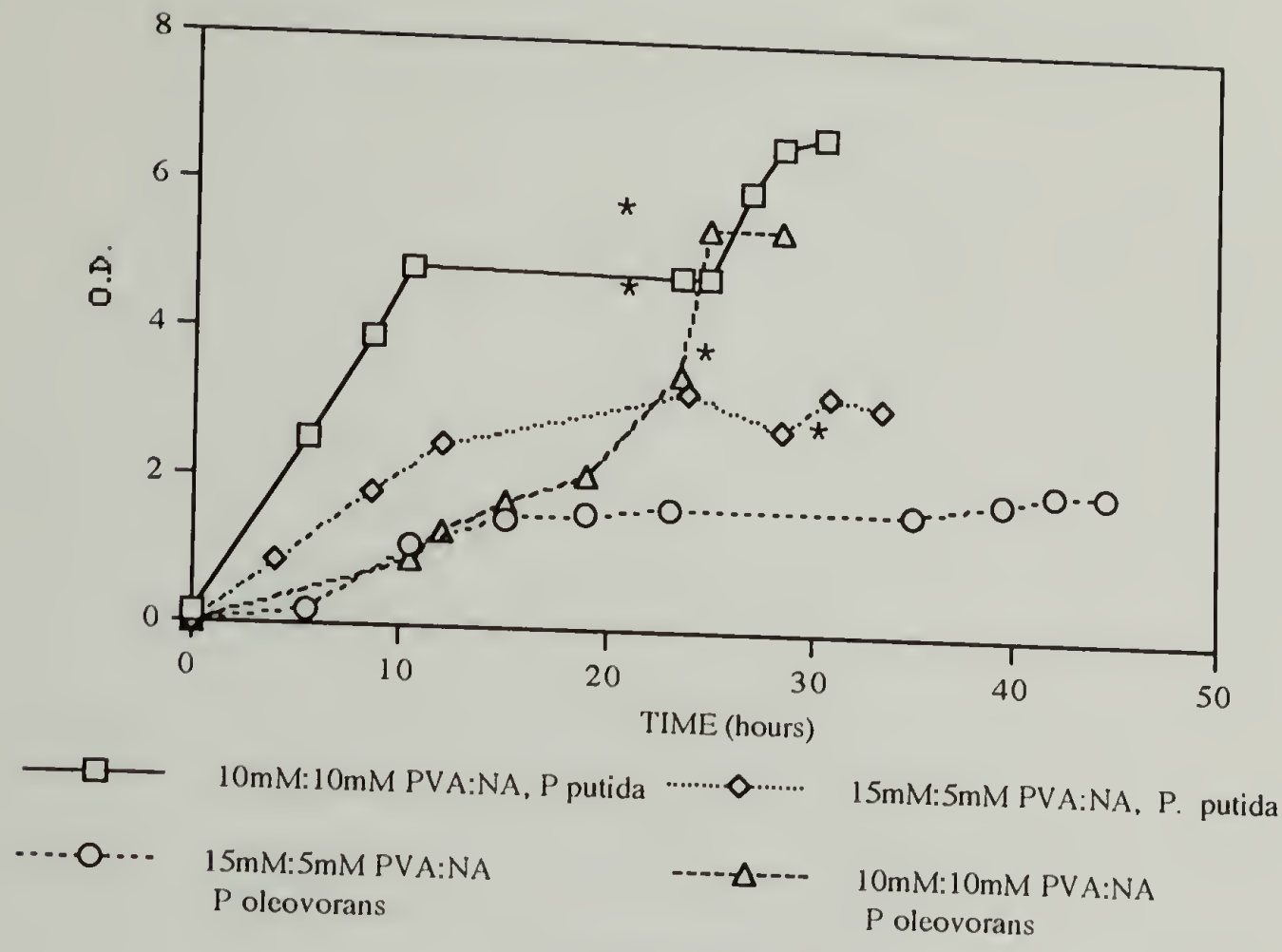


Figure 4.8 Growth curves obtained when *P. oleovorans and P. putida* were grown on various different ratios of NA and PVA

Table 4.3 Comparison of growth conditions and results obtained with *P. oleovorans and P. putida*

Substrate	Harvest Time, hours	O.D. at Harvest	Cell Yield, g/L	Polymer Yield, (g/L)	Polymer Yield, %DW[a]	% Phenyl-Containing Repeating Units
P. oleovorans						
20mM PVA	70	1.6	0.5	0.19	20	100
20mM NA	24	3.0	1.0	0.20	20	/
10mM NA : 10mM PVA	24	2.9	1.9	0.37	20	45[c]
15 mM PVA 5 mM NA	45	2.0	0.81	0.10	13	86[c]
P. putida						
40 mM NA	24	3.6	1.7	0.35	20	/
20 mM NA	28	3.5	1.6	0.10	6.0	/
20 mM NA[b]	24	1.4	0.59	0.16	17	/
20 mM PVA	134	2.6	0.90	0.16	37	100
10 mM PVA 10 mM NA	31	6.8	2.2	0.96	43	22[c]
15 mM PVA 15 mM NA	34	3.1	1.1	0.24	22	79[c]

a:Polymer yield based on cell dry weight

b: P. putida was grown on E* Media

c: These polymers are all presumably a mixture of PHN and PHPV

4.4 Conclusions

The yield of PHN was increased by multiple feeding (either manual or automatic). The same type of multiple feeding experiments were carried out using PVA as the sole carbon source, but the bacteria grew so slowly that it was difficult to distinguish changes in dissolved oxygen (D.O.) from routine instrument drift on the chart recorder used to monitor the D.O.. The maximum O.D. achieved using the multiple feeding technique with PVA as the carbon source was 1.9 as compared to an O.D. of 1.5 when the cultures were fed once and the polymer yield increased from 0.1g/L to 0.12g/L.

Prefeeding the bacteria a number of less expensive carbon sources (both polymer and non-polymer producing substrates) prior to the addition of PVA did not result in a significant increase in the amount of PHPV produced.

When *P. oleovorans* was grown in rich media, a maximum O.D. of 6-7 was achieved in 8.5 hours. Resuspending these cells in E* media containing PVA as the carbon source did not result in a further increase in O.D., and the polymer yield was the same as in the case where the cells are grown on the minimal E* media from the start of the experiment. Changing the substrate or media did not result in an increase inn the amount of PHPV produced

P. putida BM01 proved to be superior to *P. oleovorans* in that it produced a higher O.D., higher cell yield, and a higher polymer yield regardless of the substrate on which it was grown.

4.5 References

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CHAPTER 5

PRODUCTION OF CRYSTALLINE PHA's CONTAINING PHENYL GROUPS

5.1 Introduction

Poly-3-hydroxyphenylvalerate (PHPV) is one of the most interesting polymers produced by P. *oleovorans* [1]. Its Tg of 19°C is the highest of all the biopolymers produced by *P. oleovorans*. It is a homopolymer, but it does not exhibit crystallinity, which is unusual because the polymer is a highly ordered structure. Each repeating unit of the polymer has a chiral center at the 3 position and all repeating units are in the [R] configuration [2]. Therefore, some degree of ordered packing was expected. The main objective of this section of the dissertation research program was to produce a crystalline phenyl-containing polyester.

Two separate approaches were undertaken to try to achieve this goal:

(1) Various different annealing techniques were performed on PHPV to try to induce crystallinity.

(2) Modified phenyl-containing substrates were synthesized and fed to *P. oleovorans* either as a sole carbon source or cofed along with a 'good' feed source such as NA.

The second approach was based upon the results obtained by Lenz and coworkers [3-5] who reported that polymer formed by polymerizing p-methyl-α-styrene had a higher degree of crystallinity than the polymer with the same degree of tacticity obtained from α-methylstyrene. For this reason, a series of 5-phenylvalerie acid compounds was synthesized in which the para position was substituted with various groups. These modified substrates were fed to *P. oleovorans* either as a sole carbon source or cofed with a 'good' carbon source such as NA.

A comparison of the amount of poly-3-hydroxyphenylvalerate (PHPV) obtained using the microorganisms *P. putida* and *P. oleovorans* was reported in the previous chapter. *P. putida* produced higher cell yields and higher polymer yields of PHPV. For this reason, 5-(4'-tolyl)valeric acid, TVA, one of the modified phenyl-containing substrate which proved most successful with *P. oleovorans,* was also fed to *P. putida* both as a sole carbon source and cofed with nonanoic acid.

5.2 Experimental

5.2.1 Annealing Studies on PHPV

The different annealing techniques performed are described below:

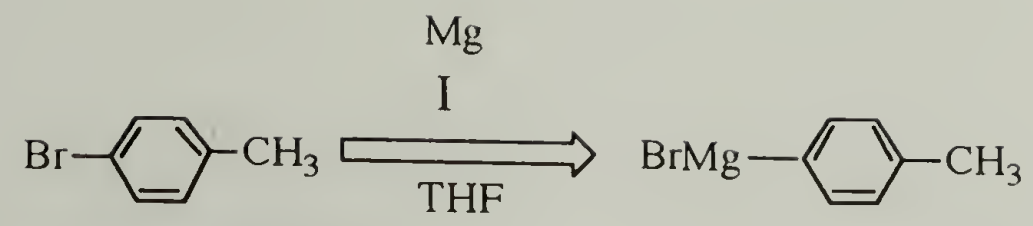
(1) In order to slow down the rate of polymer precipitation, rather than precipitating PHPV in rapidly stirring methanol, the polymer was solution cast from a 10% solution of chloroform in a chloroform saturated environment.

(2) Samples of PHPV were annealed in the DSC at two temperatures, 75°C and 160°C for 16 hours.

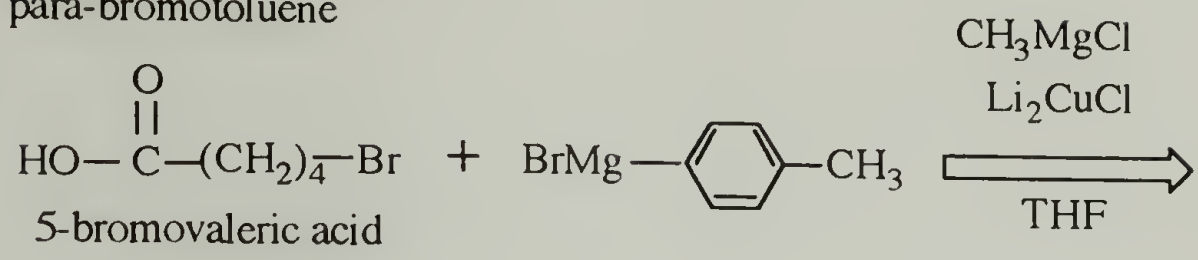
(3) PHPV samples were heated for 16 hours in solvents in which they became swollen but did not dissolve. The solvents used were hexane at 60°C and n-butanol at 100°C.

5.2.2 Synthesis of Substituted Phenyl-Containing Substrates

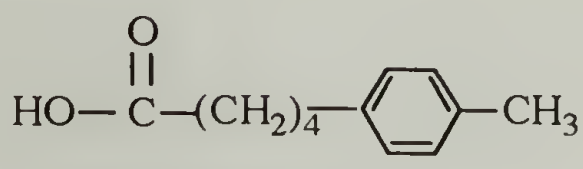
A Grignard reaction similar to that used by Fritzsche and coworkers was used to prepare these substrates [6,7] A typical example is the synthesis of 5-(4'-tolyl)valeric acid (TVA), a schematic of which is shown below.



para-bromotoluene



5-bromovaleric acid



5-(4'-tolyl)valeric acid, TVA

For this synthesis, 20g (0.120M) of 5-bromovaleric acid were dissolved in 160mls of anhydrous tetrahydrofuran (THF) and cooled to -20°C under a dry argon atmosphere. 45mls of 3M methylmagnesium bromide (MeMgBr) were added dropwise keeping the temperature below -15°C. The solution was stirred for 15 minutes and then 12mls (0.120M) of Li_2CuCl_4 was added. A Grignard reagent prepared from 24g (140mM) of 4-bromotoluene and 3.4g (140mM) of magnesium in 150 mls of THF was added dropwise to the flask, keeping the temperature between -20 and -25°C. The mixture was allowed to warm to room temperature and was left stirring for 20 hours, after which it was poured into a 1L solution of 20% H_2SO_4 in ice cold water. The aqueous phase was saturated with NaCl and extracted with ether. The combined organic layers were extracted with a solution of 50g of KOH in 100mls of water. When these extracts were acidified by pouring into a 1L solution of 20% H_2SO_4 in ice cold water, a white powder precipated out of solution. This white powder was removed from the solution by filtering through Whatman 10 filter paper. The 5-(4'-tolyl)valeric acid was purified by dissolving it in chloroform and filtering the mixture through a sintered glass funnel. The filtrate was dried over sodium sulfate for 30 minutes after which it was filtered again. Hexane was added until the solution became cloudy, and the mixture was stored at -4°C for 16 hours. The solid which precipitated was

removed by filtering. The product was purified in this manner three times. Yield 4.04g (17.4%). Melting Point 76-78^{0}C. ^{1}H NMR (200MHZ, d-acetone, TMS.) δ = 1.5–1.7 (m, 4H, H–2, H–3), 2.2–2.4 (m, 4H H–5, H–1), 2.6 (t, 4H, 2H, H-4), 7.05 (s, 4H, Ha, Hb, Hc, Hd).

5-(4'-biphenyl)valeric acid (BPVA) This substrate was prepared as described using 32.6g (0.140M) of 4-bromobiphenyl instead of 4-bromotoluene. Yield 4.11g (17.6%). Melting Point 76-78^{0}C.^{1}H NMR (200MHZ, d-acetone, TMS.) δ = 1.5–1.7 (m, 4H, H–2, H–3), 2.25–2.4 (t, 2H H–1), 2.6–2.8 (t, 2H, H-4), 7.2-7.6 (m, 9H, Ha, Hb,Hc, Hd,He, Hf, Hg, Hh, Hi,)

5-(4'-ethylphenyl)valeric acid (EPVA). This substrate was prepared as described above using 25.9g (0.14M) 4-bromoethylbenzene instead of 4-bromotoluene. Yield 4.75g (19.2%). ^{1}H NMR (200MHZ, d-acetone, TMS.) δ = 1.1–1.5 (t, 3H, H–6), 1.55–1.8 (m, 4H H–2, H–3), 2.25–2.5 (t, 2H, H-1), 2.5–2.9 (m, 4H, H-4,H-5), 7-7.5 (s, 4H, Ha, Hb,Hc, Hd).

8-(4'-tolyl)octanoic acid (TOA) This substrate was prepared as described above using 26.8g (0.12M) 8-bromooctanoic acid instead of 5-bromovaleric acid Yield 3.9g (13.8%) bp 147-149^{0}C/ 11 Torr. ^{1}H NMR (200MHZ, d-acetone, TMS.) δ = 1.45–1.6 (m, 8H, H–3, H–4, H–5, H–6), 1.65–1.9(m, 4H H–2, H–7),2.25 (s, 3H, H–9), 2.35–2.5 (t, 2H, H-1), 2.7-2.85 (t, 2H, H-8), 7.3-7.5 (m, 4H, Ha, Hb,Hc, Hd)

5.2.2.1 Monomer Characterization

The melting points of the substrates synthesized were determined using an Electrothermal™ melting point apparatus. ^{1}H and ^{13}C spectroscopy were performed as described in Chapter 2. Elemental analysis was performed at the Microanalysis laboratory at the University of Massachusetts, Amherst.

The carbon substrates prepared and the anticipated polymers are shown below

5.2.3 PHA's from Substituted Phenyl Containing Substrates

5.2.3.1 PHA Biosynthesis

Stock cultures of *P. oleovorans* (ATCC 29347) were used in these experiments. *P. oleovorans* was grown on 10mM of carboxylic acids according to the method described in Section 2.2. 1L cultures containing the various substituted carboxylic acid as a carbon substrate were used both as the sole carbon source and cofed with nonanoic acid. 100mls from a 250ml culture grown on nonanoic acid was used as a preculture to inoculate the 1L cultures.

5.2.3.2 Polymer Characterization

The polymers produced were characterized using the following techniques: ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR), differential scanning calorimetry (DSC), and gas chromatography (GC) of methanolyzed polymers
All of these techniques and sample preparation required are described in Section 2.2

Wide angle X-Ray scattering (WAXS) patterns were obtained as described in Section 2.2. The degree of crystallinity of the PHN and PHTV polymer were determined using a Siemens D500 diffractometer. The scattering angle 2θ was varied from 5 to 35, and the corresponding intensity recorded. Intensity versus 2θ were plotted for PHN (both in its semicrystalline and amorphous forms) and for the blend of PHN/PHTV polymer (again both in its semicrystalline and amorphous forms), and are show in Figure 5.8a and b respectively. The degree of crystallinity Xc was determined from Bragg's Law as follows

Bragg's Law $2d\sin\theta=n\lambda$ and $n=1$ so $d=\lambda/2\sin\theta$

$s=1/d$, so $s=2\sin\theta/\lambda$

$$Xc = \frac{\Sigma S^2 Ic(s)\, ds}{\Sigma S^2 I(s)\, ds}$$

where d is the periodicity of lattice, s is 1/d, I(s) is the intensity of the coherent scattering from a specimen at the point s in reciprocal space, Ic(s) is the part of the intensity at the

same point that is concentrated in the crystalline peaks and Xc is the fraction of crystalline material in the specimen.

I(s) versus s^2 was plotted for PHN and for the blend of PHN/PHTV when the polymer(s) were in both the amorphous and semicrystalline form. The degree of crystallinity was calculated by determining the area under the curve, which resulted from scattering by the crystalline component, and dividing it by the total area under the curve. Amorphous samples of PHN and of the mixture of PHN/PHTV, were obtained by melting the samples at 10°C above their melting points and taking their X-Ray diffraction pattern before recrystallization had occurred.

5.3 Results and Discussion

5.3.1 Annealing Studies on PHPV

Characterization of PHPV by both wide angle X-ray scattering (WAXS), and differential scanning calorimeter, DSC (Figure 5.1) showed that the polymer precipitated from rapidly stirring methanol was not crystalline. The endotherm at -50°C in the DSC thermogram in Figure 5.1 was due to the inorganic antifoaming agent, which was added during fermentation and remained with the polymer throughout the extraction procedure. None of the DSC thermograms of annealed PHPV (Fig. 5.2) exhibited endotherms which could be assigned as melting transitions, indicating that the polymer samples were all completely amorphous.

5.3.2 PHA's from Substituted Phenyl-Containing Substrates

P. oleovorans was grown on 5-(4'-tolyl)valeric acid (TVA), 5-(4'-ethylphenyl)valeric acid (EPVA), 5-biphenylvaleric acid (BPVA), 5-naphtylvaleric acid (NVA), or 8-(4'-tolyl)octanoic acid (TOA) as the sole carbon source. The growth curves for *P. oleovorans* grown on modified phenyl-containing substrates as a sole substrate are shown in Figure 5.3. The bacterial growth was slow, the maximum O.D. (0.8) was

achieved when TOA was used as a substrate. However, no polymer was obtained when the culture grown on TOA was extracted.

BVA and NVA were hydrophobic and did not dissolve in the water based E* media, consequently these substrates were not metabolized by *P. oleovorans*. Various attempts which were made to dissolve these two substrates in water, included changing the pH from 7 to 9 and dissolving the substrates in 10 mls of dimethylsulfoxide (DMSO) or methanol before adding the solution to the E* media. None of these attempts increased the substrate solubility to such an extent that *P. oleovorans* was able to incorporate it into the polymer backbone. No attempt was made to extract the cultures which were grown TVA, PEBVA, BVA or NVA as the final O.D. achieved was very low.

5.3.3 Cofeeding of Substituted Phenyl-Containing Substrates

When *P. oleovorans* was grown on substituted phenyl-containing substrates as a sole carbon source, the maximum O.D. achieved was low, so, the next step was to cofeed these substrates along with a 'good' carbon source; that is, one on which the bacteria grow rapidly and produce polymer. Therefore the object of cofeeding the substituted phenyl-containing substrates with 'nonanoic' acid was to increase the yield of the 'poorer' phenyl-containing polymer.

All of the modified phenyl containing substrates were cofed along with NA and in addition TVA was also cofed with PVA. The harvest time, O.D. at harvest, cell yield, % polymer (based on cell dry weight) and the % phenyl containing polymer are given in Table.1. In the cases where TOA or PEPVA and NA were cofed to *P. oleovorans* , the bacteria only metabolized the NA, resulting in the production of PHN, which is the polymer which would be obtained if NA were the sole carbon source. When BVA was cofed with NA, phenyl groups were detected in the polymer produced. However, subsequent reprecipitation of this polymer reduced the amount of phenyl groups detected from 18 to 5%, indicating that the phenyl groups detected were not polymer but contamination by the BVA feed substrate.

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An interesting result was that obtained when a 1L culture of TVA was inoculated with 100mls of a preculture grown on NA. The aim of the experiment was to produce 100% poly-3-hydroxy-5-(4'-tolyl)valerate, PHTV, but PHN was present from the 100mls of preculture, grown on NA which was used to inoculate the 1L culture which contained TVA as a sole carbon source. From the ^{1}H NMR spectrum (Figure 4) it was determined that 40% of the repeating units obtained were PHTV. The ^{13}C NMR spectrum was assigned as shown in Figure 5

The DSC thermogram of the polymer produced (Figure 6) clearly shows two glass transition temperatures Tg's, one at -37°C which corresponds to the Tg of PHN and one at 17°C, which was assigned as the Tg of PHTV. The thermogram also exhibited two melting transitions, Tm's, one at 48°C which corresponds to the Tm of PHN, and the other at 95°C which was assigned as the Tm of PHTV, proving that PHTV is a crystalline polymer

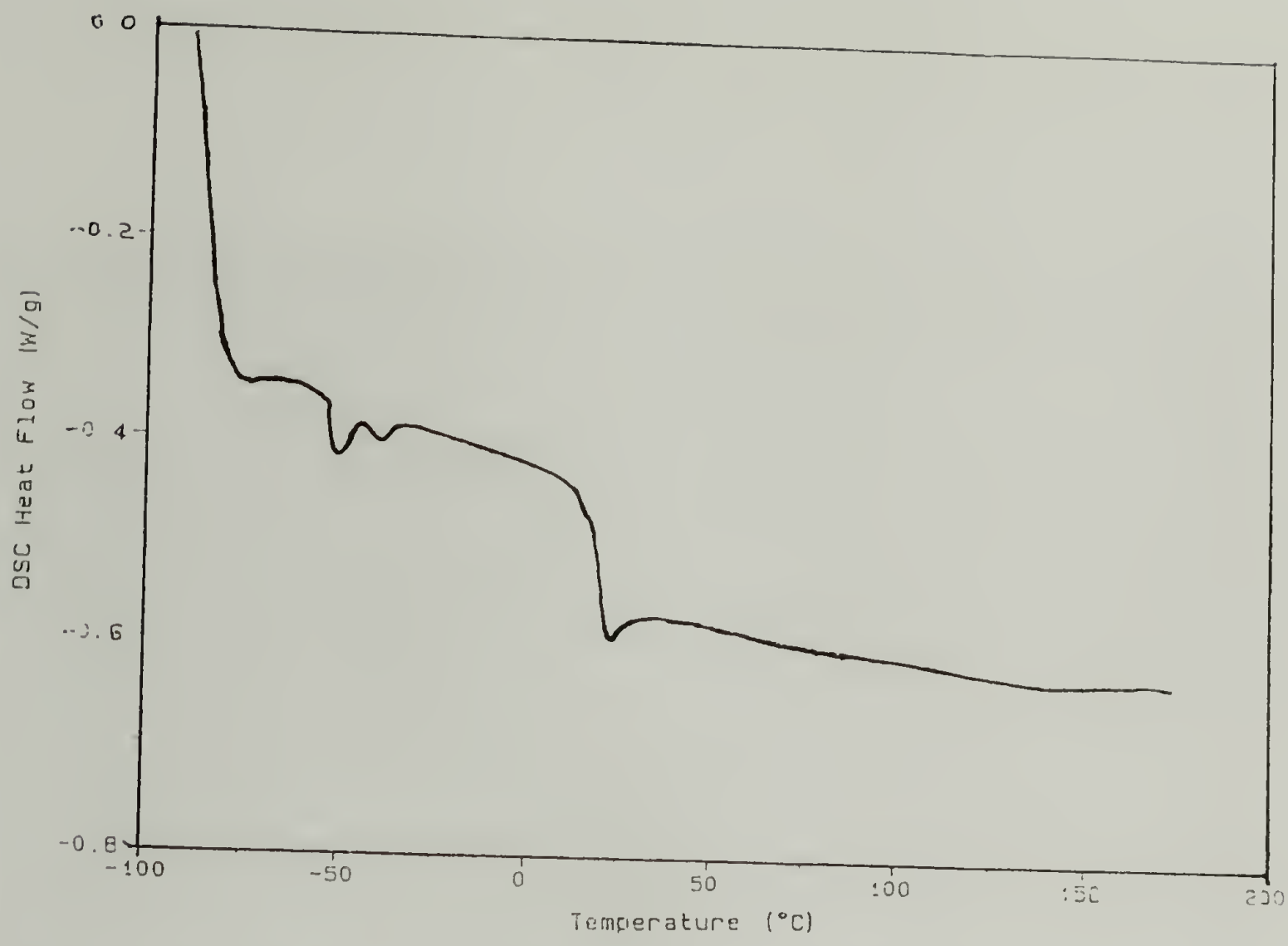


Figure 5.1 DSC thermogram of poly-3-hydroxyphenylvalerate (PHPV)

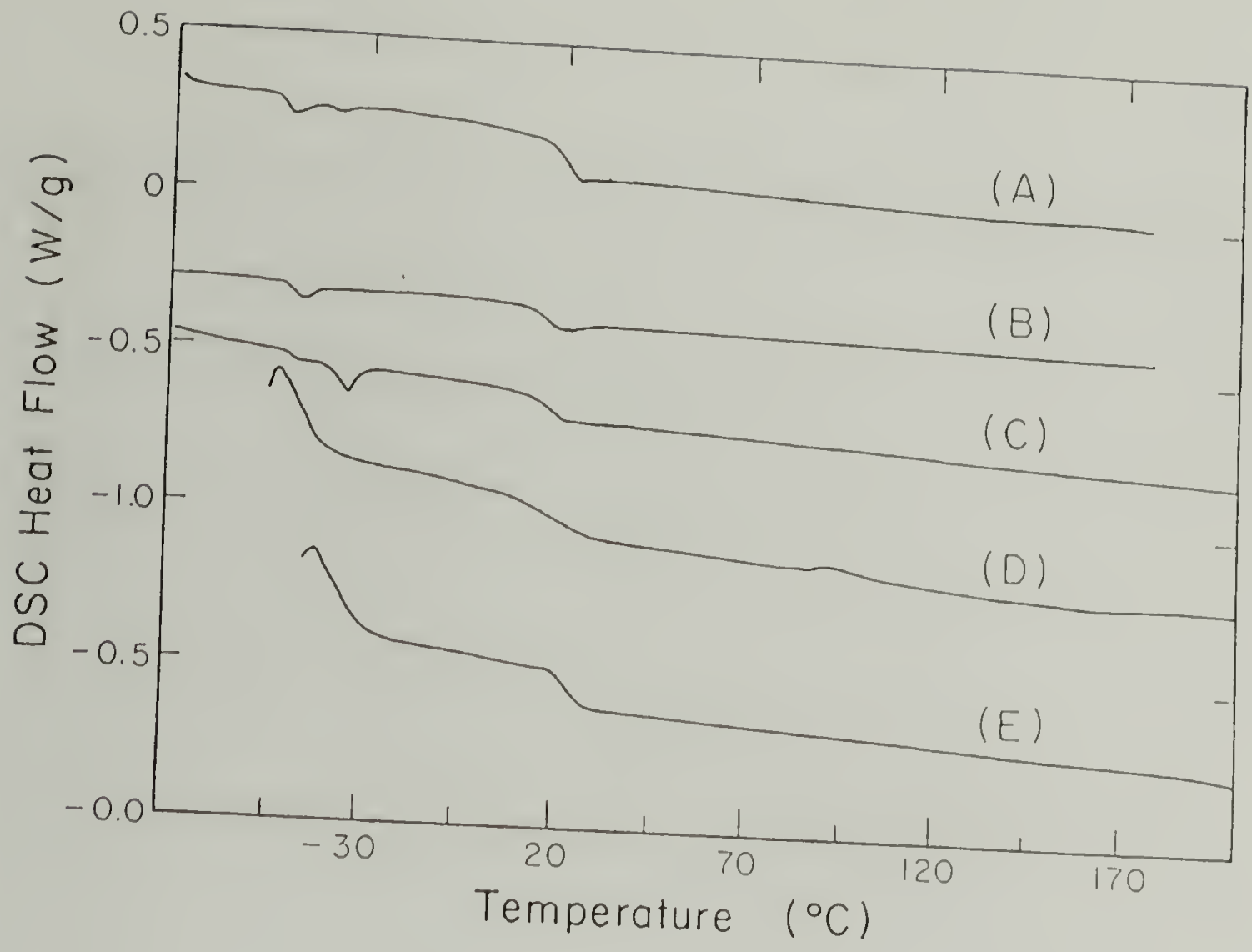


Figure 5.2 DSC thermograms from various annealing experiments performed on PHPV: (A) PHPV precipitated in methanol, (B) PHPV solution cast in 10% chloroform solution, (C) PHPV annealed at 160^{0}C for 16 hours, (D) PHPV film annealed in n-heptane for 16 hours at 100C, (E) PHPV annealing in the DSC for 16 hours at 75^{0}C

Table 5.1 Growth conditions and results obtained when *P. oleovorans* was cofed substituted phenyl-containing substrates and NA or PVA

Substrates[a]	Harvest Time, hours	O.D at harvest	Cell Yield, g/L	Polymer Yield % DW[b]	% Phenyl-Containing Repeating units	
					After 1st Precipitation	After 2nd Precipitation
5 mM TVA 15 mM NA	21	3.4	1.4	15	3.0	3.8
5 mM TVA: 5 mM NA	19	1.5	0.68	7.0	27	27
5 mM TVA: 5 mM PVA	46	1.2	0.45	23	64 PTVA	
5 mM BPVA: 5 mM NA	23	1.7	1.0	13	18.0	5.0
5 mM TOA: 5 mM NA	19	1.5	0.62	5.6	0	
5 mM PEBVA: 5mM NA	25	2.8	1.52	5.2	0	
10 mM TVA[c]	54	1.2	0.58	0.03	40	

a: TVA is 5-(4'-tolyl)valeric acid, NA is nonanoic acid, BPVA is 5-biphenylvaleric acid, TOA is 8-(4'-tolyl)octanoic acid, PEBVA is para-ethylbenzylvaleric acid

b: Polymer yield based on cell dry weight

c: PHN was also present due to the use of a preculture which contained NA as a carbon substrate

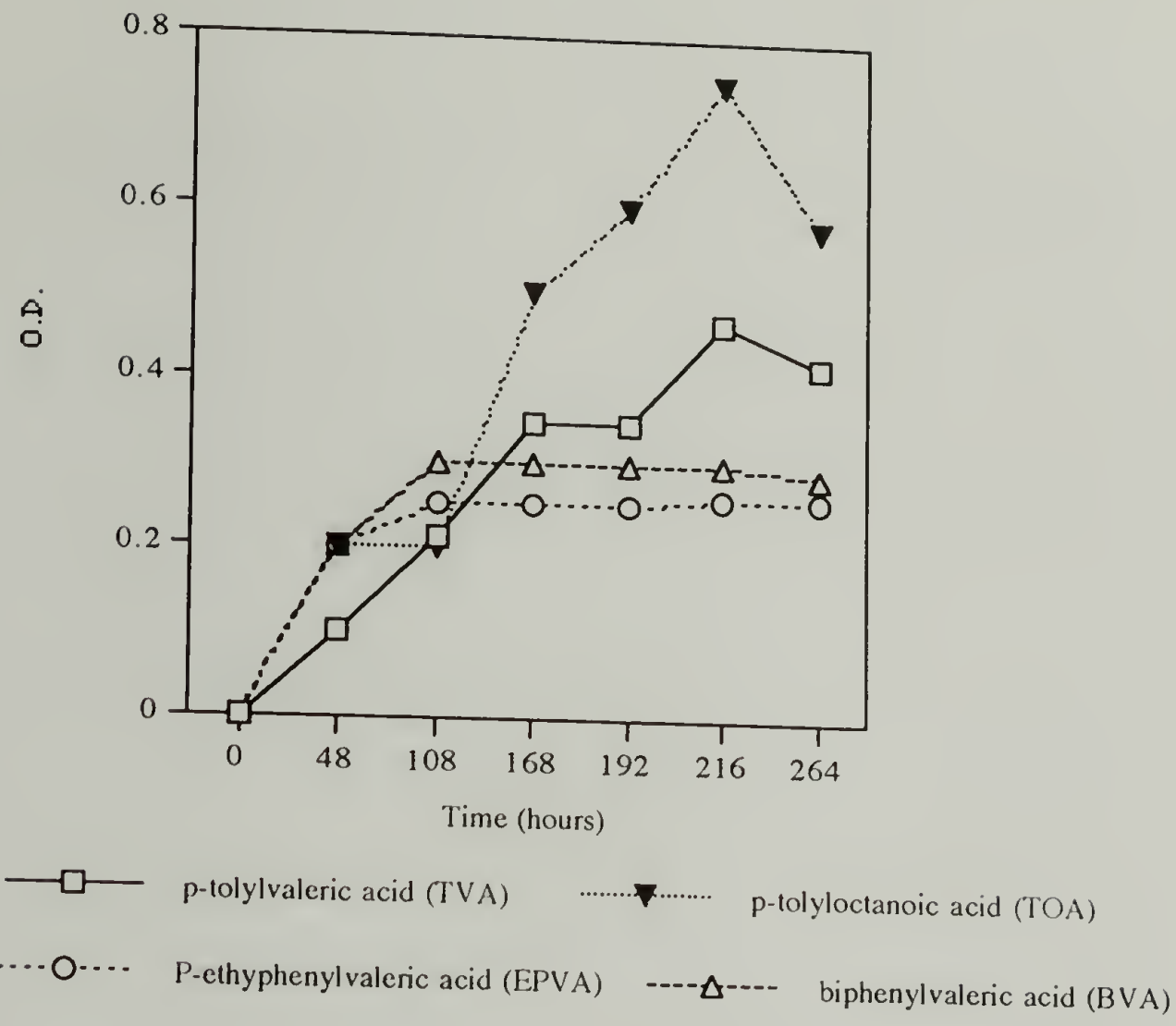


Figure 5.3 Growth curve obtained when *P. oleovorans* was grown on substituted phenyl-containing substrates as the sole carbon source

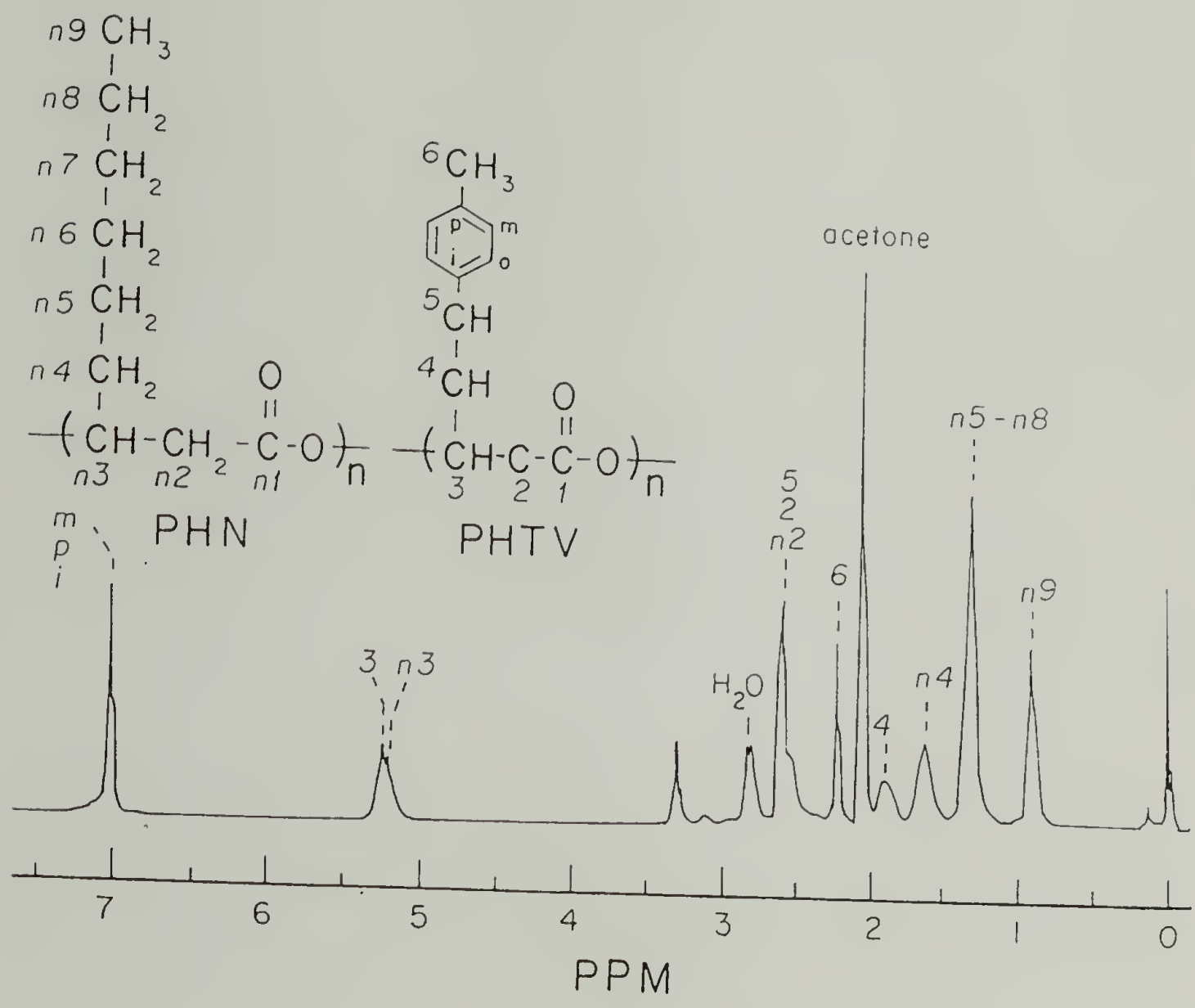
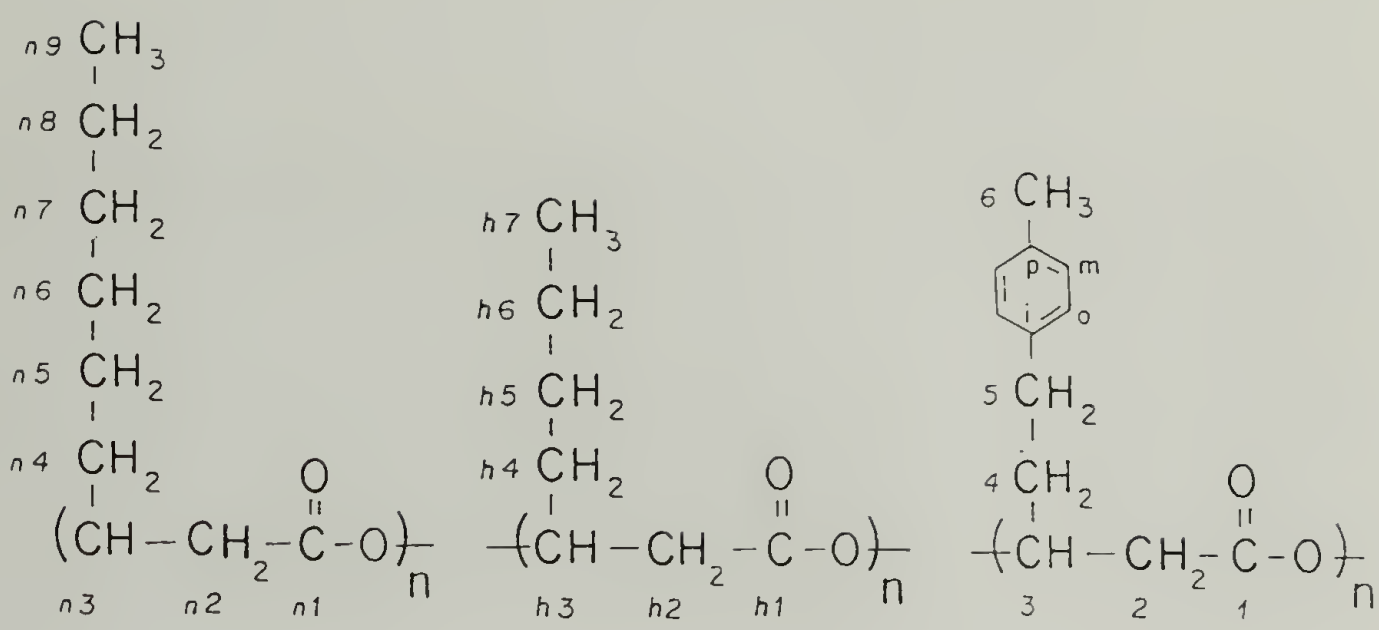


Figure 5.4 ^{1}H NMR spectrum of the polymer obtained when *P. oleovorans* was grown on TVA



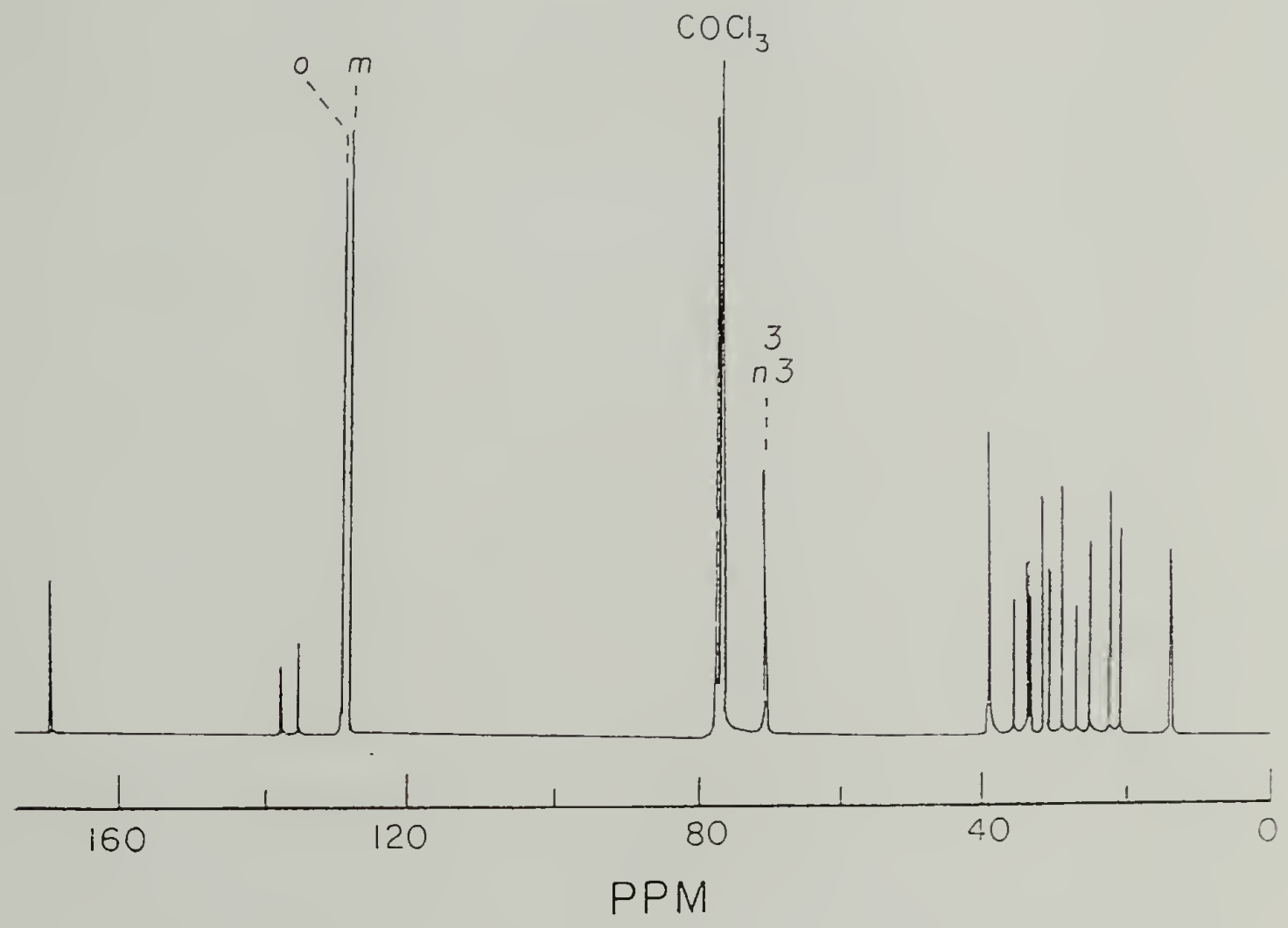
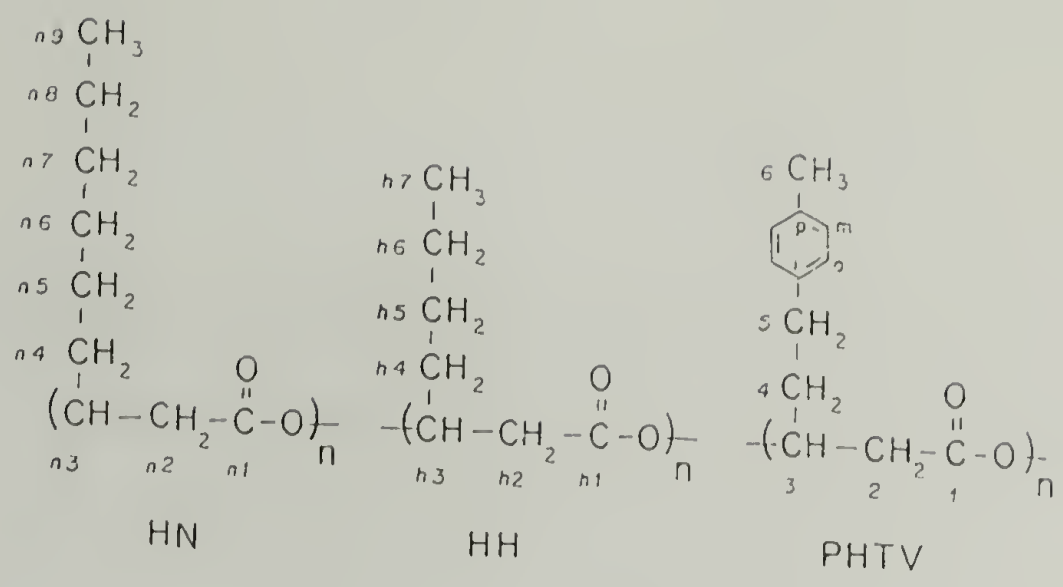
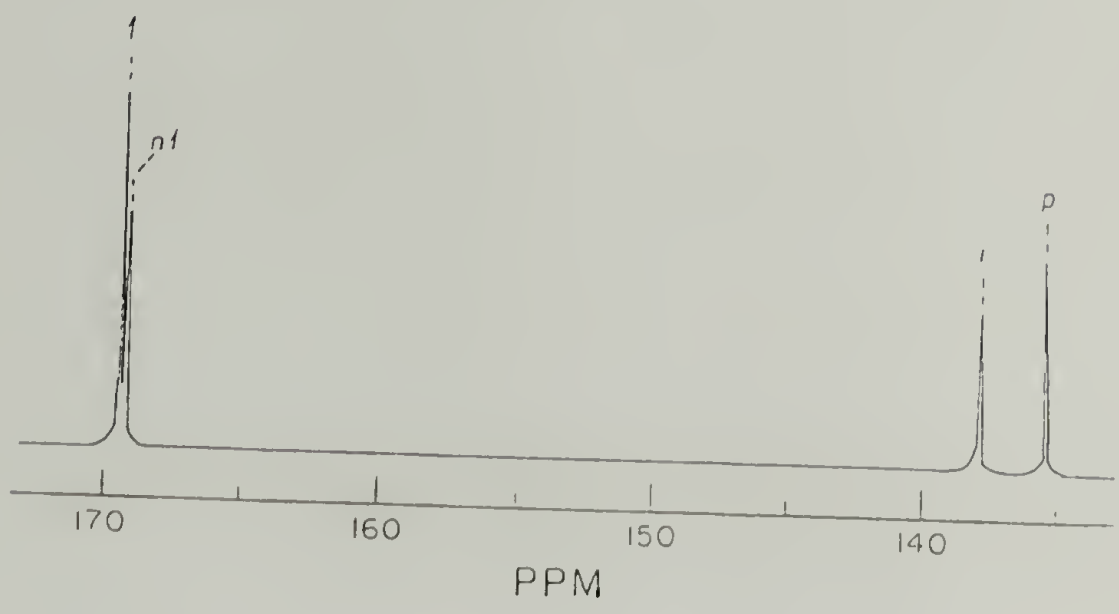


Figure 5.5 ^{13}C NMR spectrum of the polymer obtained when *P. oleovorans* was grown on TVA (continued next page)





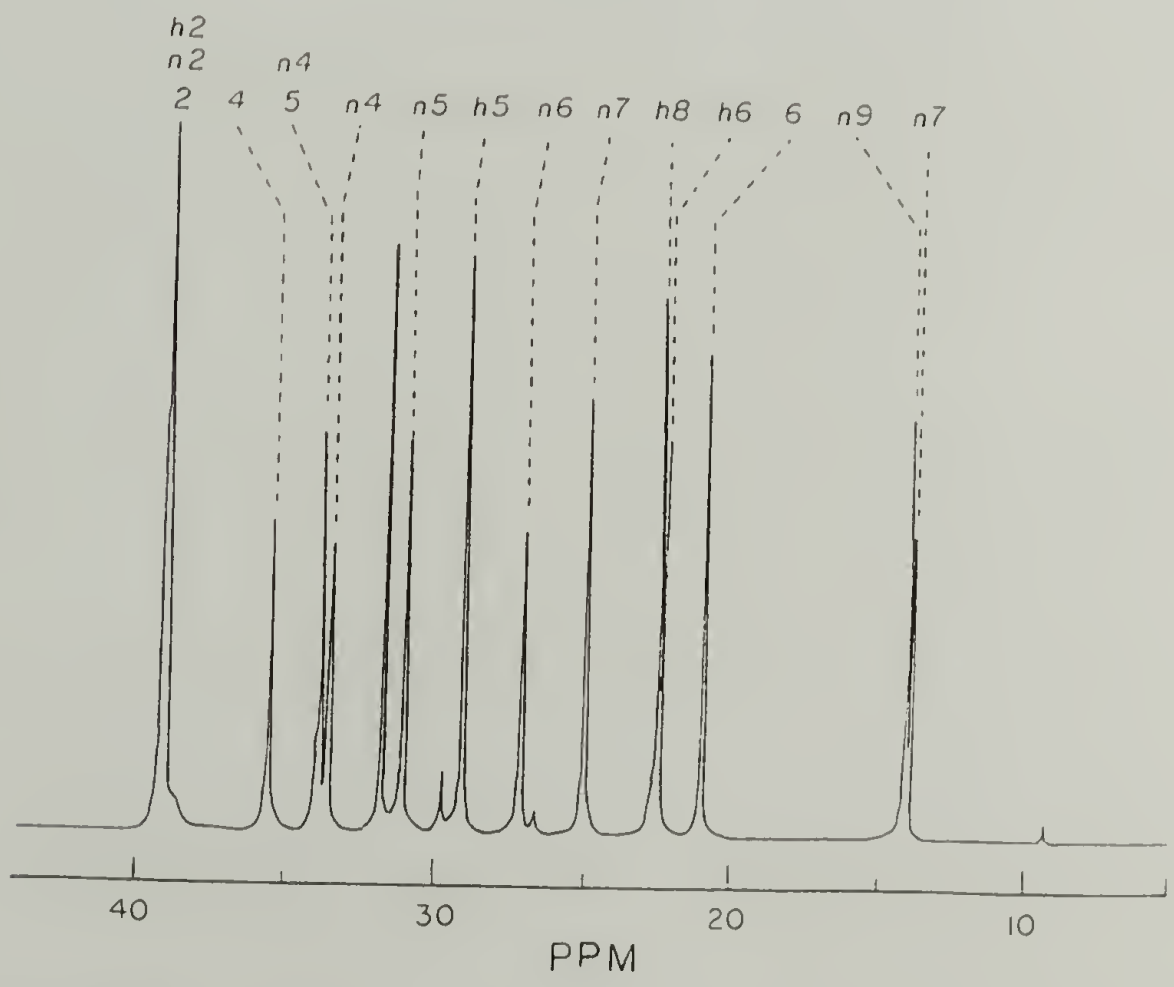


Figure 5.5 continued

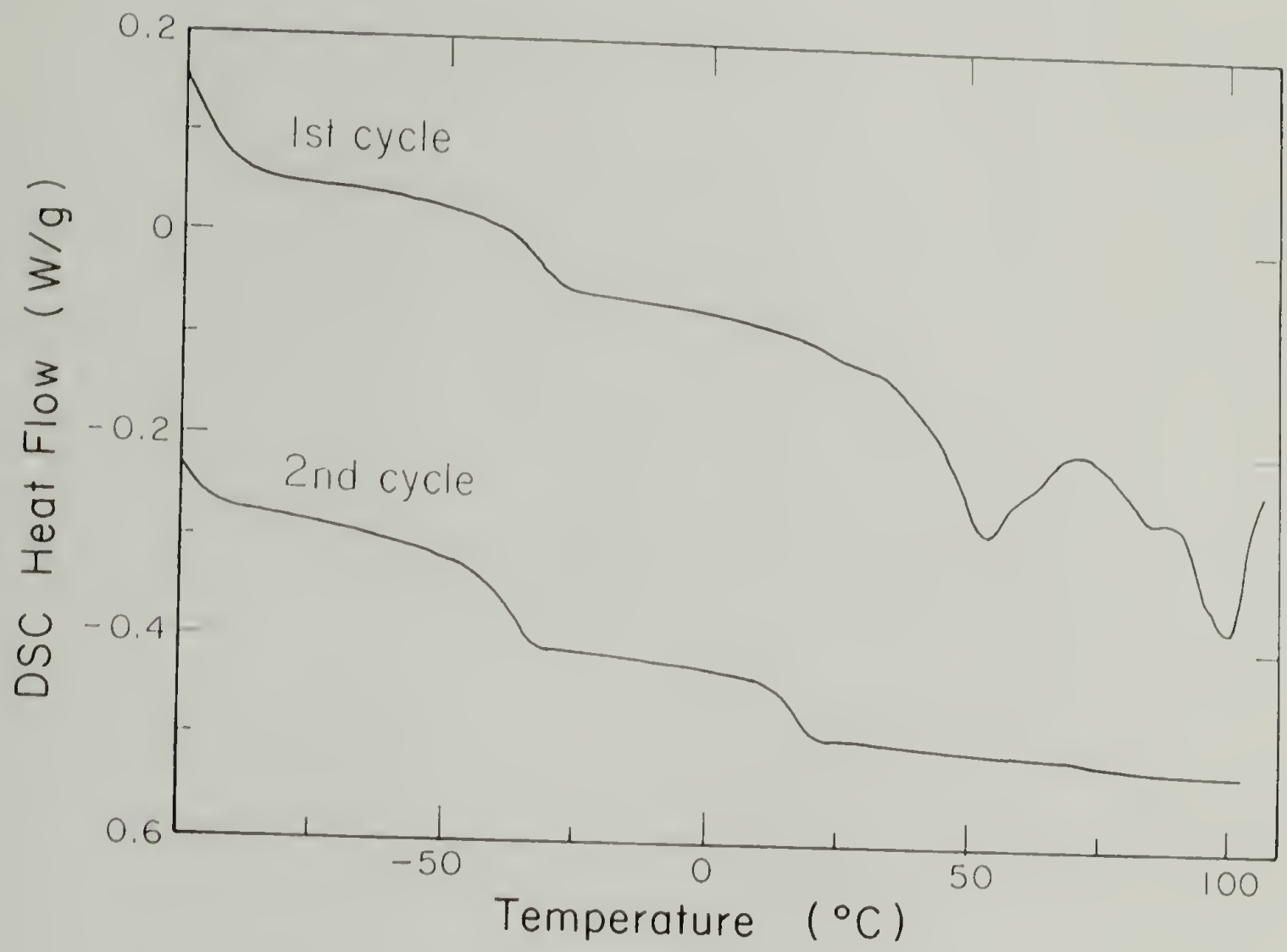


Figure 5.6 DSC thermograms of the polymer obtained when *P. oleovorans* was grown on TVA (1) first heating cycle, (2) second heating cycle

When wide angle X-ray scattering (WAXS) was performed on PHN and on a blend of PHN and PHTV, a number of distinct rings were obtained in each WAXS pattern. A comparison of the WAXS pattern obtained from PHN (Figure 5.7a) to that obtained from a blend of PHN and PHTV (Figure 5.7b) shows that PHTV and PHN do not crystallize in the same matrix. The interplanar spacing (d spacings) for PHN and PHPV were calculated as follows, and are reported in Table 5.2 and 5.3 respectively.
The distance from the sample to the camera (L) was calculated using Bragg's Law and $CaCO_3$ as a standard, knowing L and measuring r, Equation 1 was used to calculate θ, and then Equation 2 was used to calculate d as follows:
Equation 1: Tan θ = r/L, where L is the distance from the sample to the negative, and r for each ring is the distance on the WAXS negative from the center to that diffraction ring.
Equation 2: $2d \sin\theta = n\lambda$ (Bragg's Law)
by using $CaCO_3$ as a standard for which d=3.036Å, and knowing that λ= 1.542Å, then L =53.18 mm

From the value of L and by measuring the values of r for each ring (where r is the distance from the center of the diffraction pattern to that diffraction ring), d can be calculated for PHN and the blend of PHN/PHTV using Equation 1 and 2 above.

Table 5.2 r, the distance on the WAXS negative from the center to the diffraction ring, and d, the interplanar spacing for PHN

r (mm)	4.3	18.8	21.4
d (Å)	19.10	4.55	4.05

Table 5.3 r, the distance on the WAXS negative from the center to the diffraction ring, and d, the interchain spacing for a bland of PHN/PHTV

r (mm)	9.7	16.1	17.7	20.2	21.8
d (Å)	8.6	5.3	4.8	4.3	4

Marchessault and coworkers [8] carried out WAXS experiments on a number of PHA's from poly-3-hydroxybutyrate (PHB) to poly-3-hydroxydecanoate (PHD) and determined the d spacing for each polymer. The d spacings which were reported for PHN are in agreement with those reported in Table 5.2.

In order to determine the degree of crystallinity of PHTV intensity versus angle (2θ) was determined for both PHN (Figure 5.8a) and the blend of PHN/PHTV (Figure 5.8b). By determining the area under the curve of intensity versus s^2 (where s is 1/d) for both the semicrystalline and amorphous forms of each polymer, the degree of crystallinity in PHTV polymer could be determined. Unfortunately, due to the significant amount of background scatter (the sample size was only 30mg) the result was not accurate. This problem could be offset by using a larger sample size.

When the bacteria was cofed an equimolar mixture of 5-phenylvaleric acid and 5-(4'-tolyl)valeric acid, the polymer produced was 36% poly-3-hydroxyphenylvalerate (PHPV) and 64% poly-3-hydroxy-5-(4'tolyl)valerate (PHTV) as determined by ^{1}H NMR (Figure 5.9). Characterization of this polymer by WAXS and DSC indicated that it was not crystalline. The lower temperature DSC thermogram (Figure 5.10a) of the polymer produced, showed one Tg at 21°C, and a higher temperature DSC thermogram (Figure 5.10b) did not exhibit any melting. The WAXS pattern of this polymer was representative of a totally amorphous polymer.

The two possible explanations for the lack of crystallinity in this sample, are as follows;

(1) The two substrates are very similar, and it is possible that the bacteria consumed both substrates at a similar rate producing a random copolymer, which contained both 3-hydroxyphenylvalerate and 3-hydroxy-5-(4'tolyl)valerate repeating units.

(2) The bacteria produced a blend of PHPV and PHTV, but they are so similar that they form a totally miscible blend, and the presence of the non-crystalline PHPV polymer prevented the crystallization of the PHTV polymer.

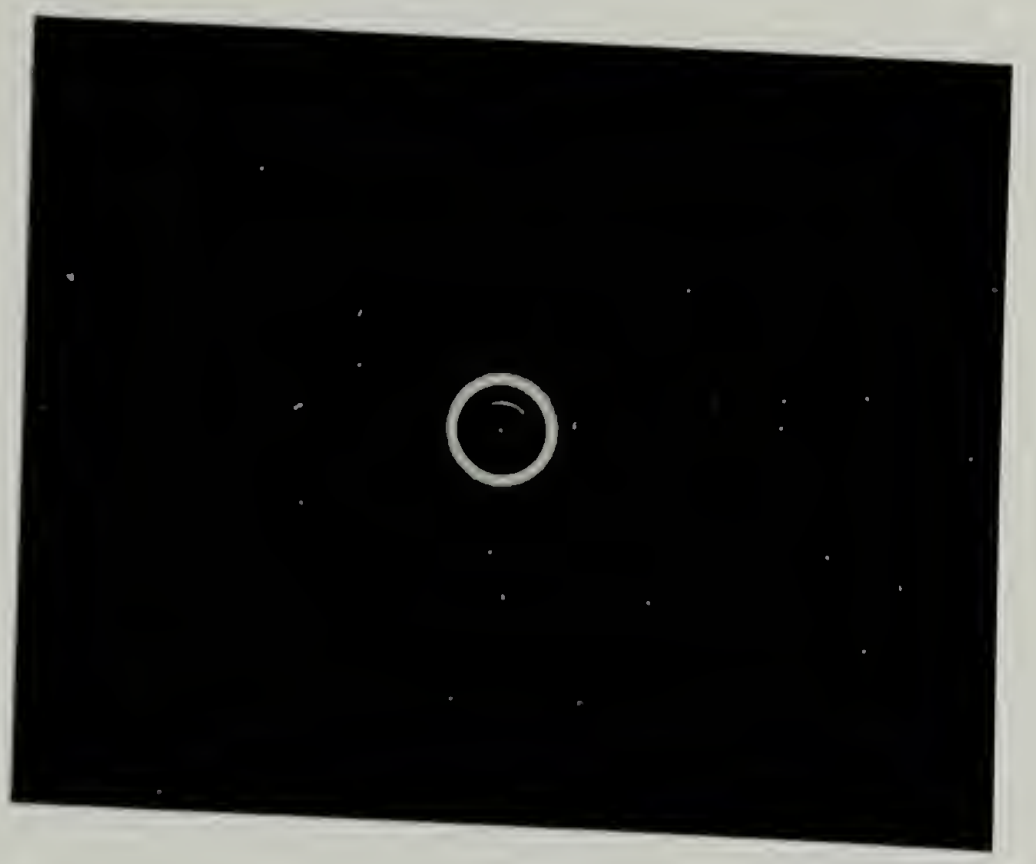
Solvent fractionation is one of the simplest method for separating polymer blends This procedure has been used to fractionate a blend of PHN and PHPV using chloroform and hexane [9]. While both polymers were soluble in chloroform, PHPV was insoluble in hexane and could be removed by centrifugation. This fractionation technique could not be performed on PHPV and PHTV as their structures are so similar that it would be very difficult to find a solvent in which one of the polymers dissolved in preference to the other.

5.3.4 p-tolylvaleric acid as a substrate for *Pseudomonas putida*

Figure 5.11 compares the growth of TVA on *P. oleovorans* and *P. putida* . Both cultures were not grown under the same conditions. The culture of *P. oleovorans* was inoculated from a plate whereas the *P. putida* culture was inoculated with a preculture which was grown on PVA. Therefore, it is not surprising that the *P. putida* grew better.

Figure 5.12 compares the growth of *P. putida* on PVA and PTVA. PVA proved to be a better substrate, as a higher O.D., cell yield, and polymer yield were obtained when it was used as a substrate. Figure 5.13 compares the growth of *P. putida* to that of *P. oleovorans* on different ratios of NA/TVA, and the results obtained are given in Table 5.4 . In all cases, *P. putida* grew better than *P. oleovorans,* and the cell yield and polymer yield were higher.

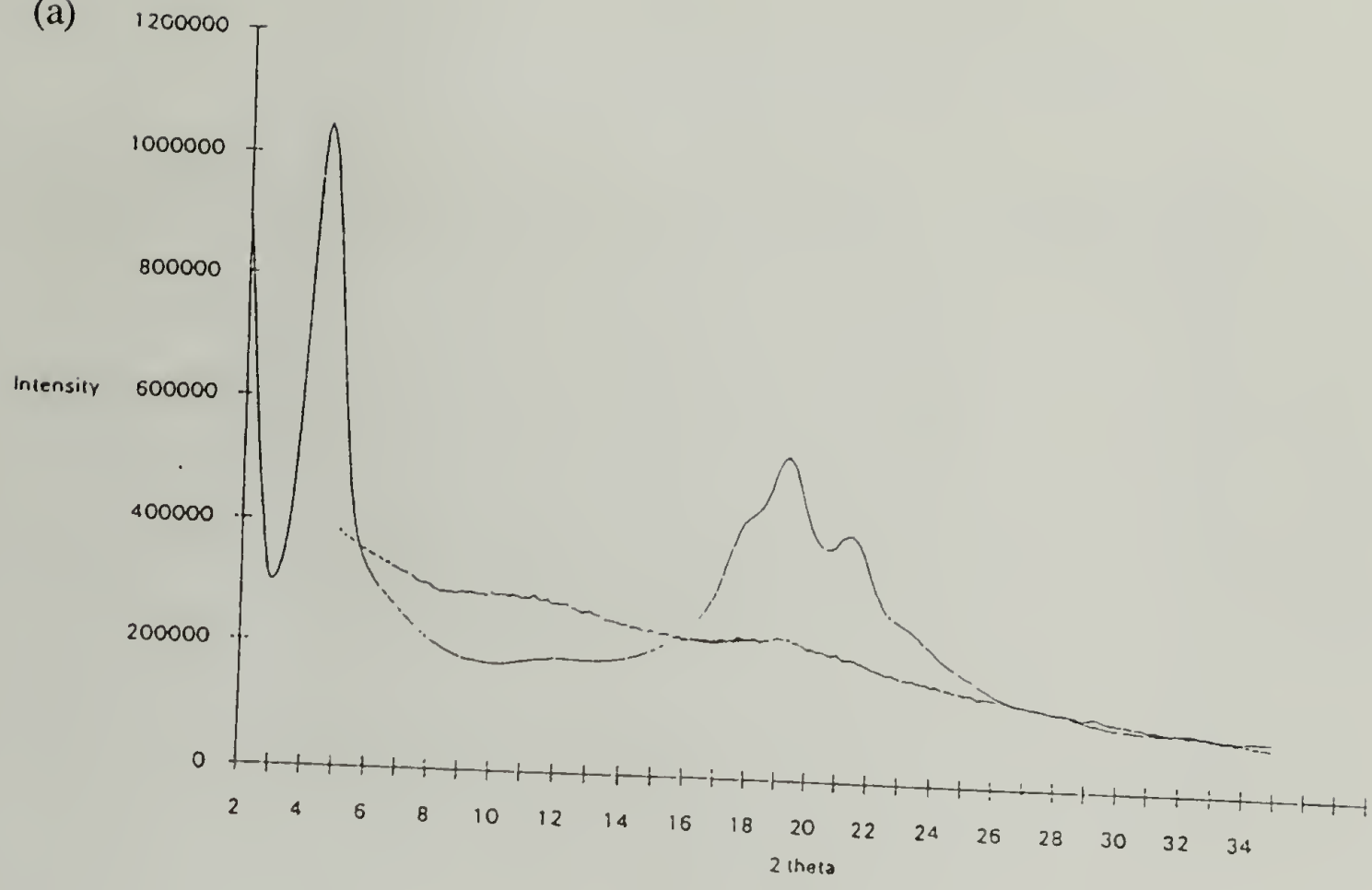
(a)



(b)

Figure 5.7 WAXS pattern of (a) PHN, (b) the mixture of PHN/PHTV

(a)



(b)

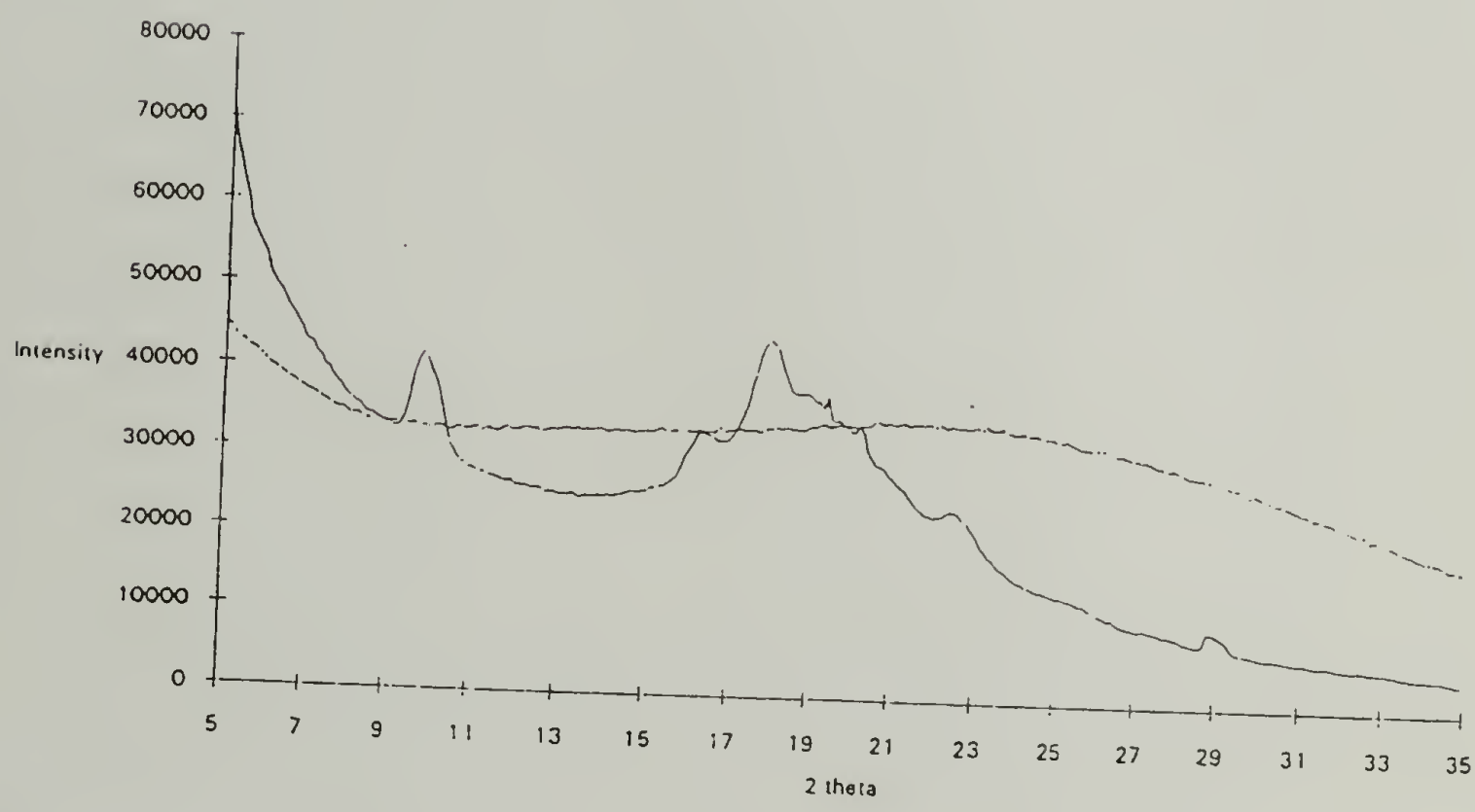


Figure 5.8 Intensity versus 2θ, from a WAXS experiment in which θ was varied and the corresponding intensity measured for (a) PHN both in a semicrystalline and an amorphous form (b) of a mixture of PHPV/PHTV both in a semicrystalline and an amorphous form

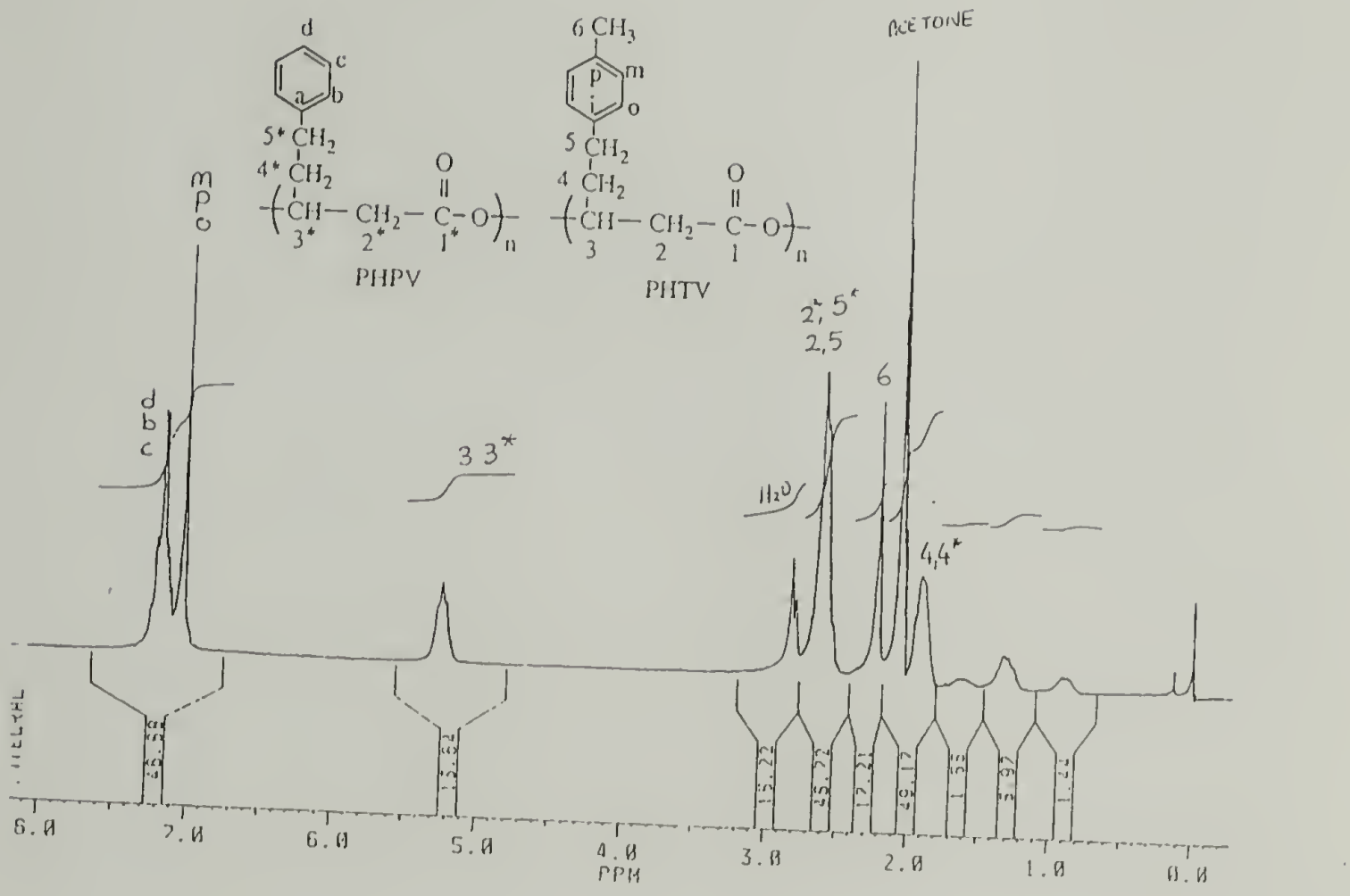


Figure 5.9 ^{1}H NMR spectrum of the polymer produced when *P. oleovorans* was cofed an equimolar mixture of PVA and TVA

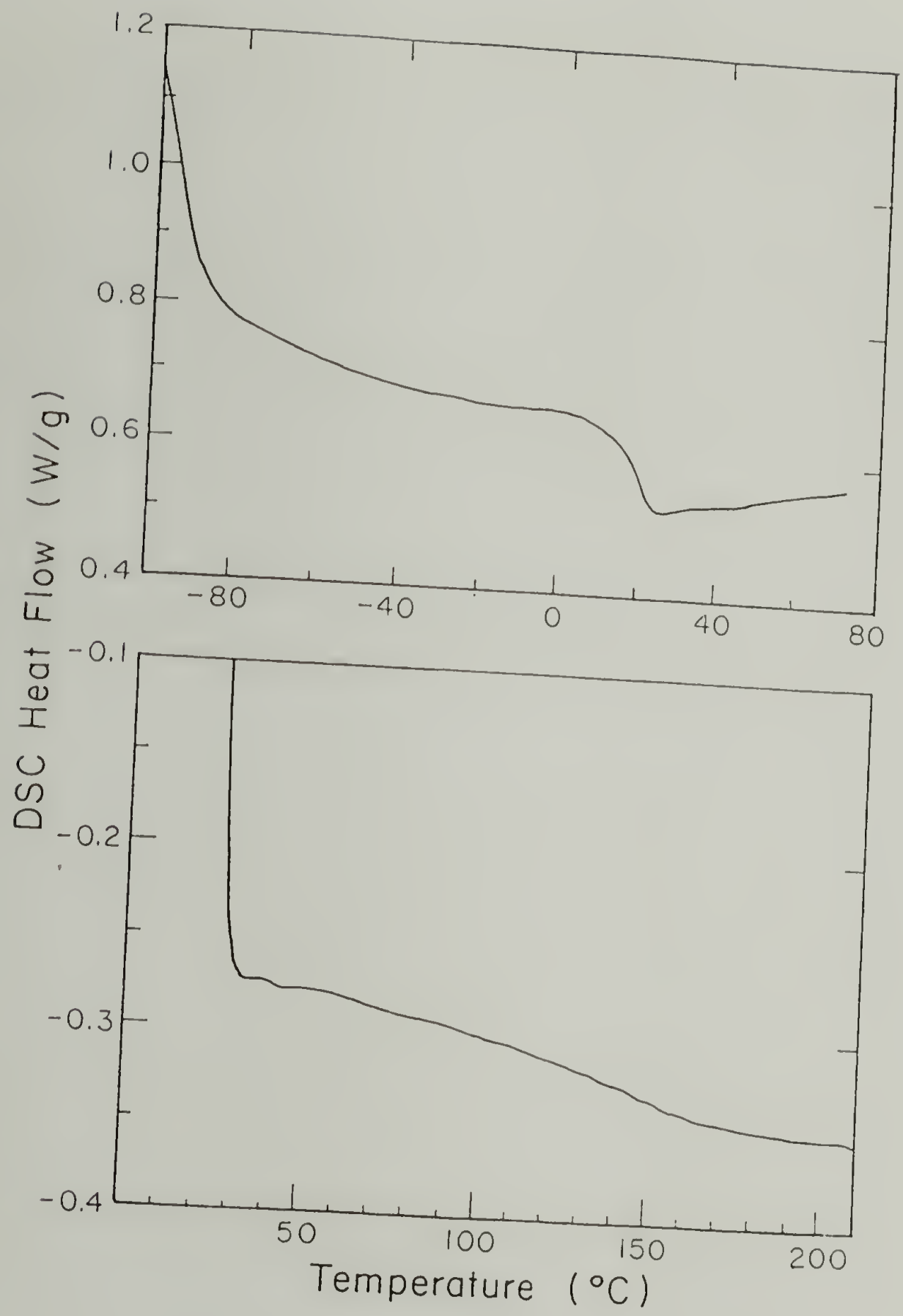


Figure 5.10 (a) Lower temperature DSC thermograms (-100^{0}C to +100^{0}C) of the polymer produced when *P. oleovorans* was cofed an equimolar mixture of PVA and TVA
(b) Higher temperature DSC thermogram (25^{0}C to 225^{0}C) of the polymer produced when *P. oleovorans* was cofed an equimolar mixture of PVA and TVA

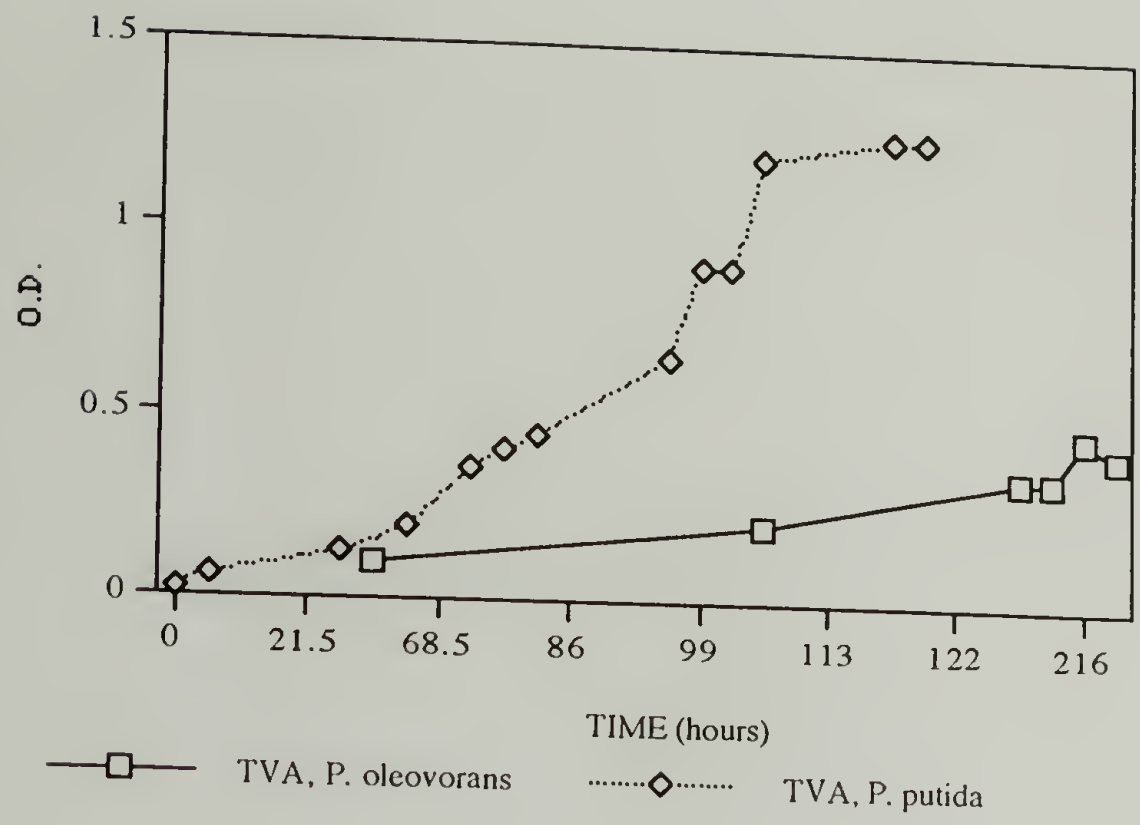


Figure 5.11 Growth curves obtained when *P. oleovorans* and *P. putida* were grown on TVA as a sole carbon source.

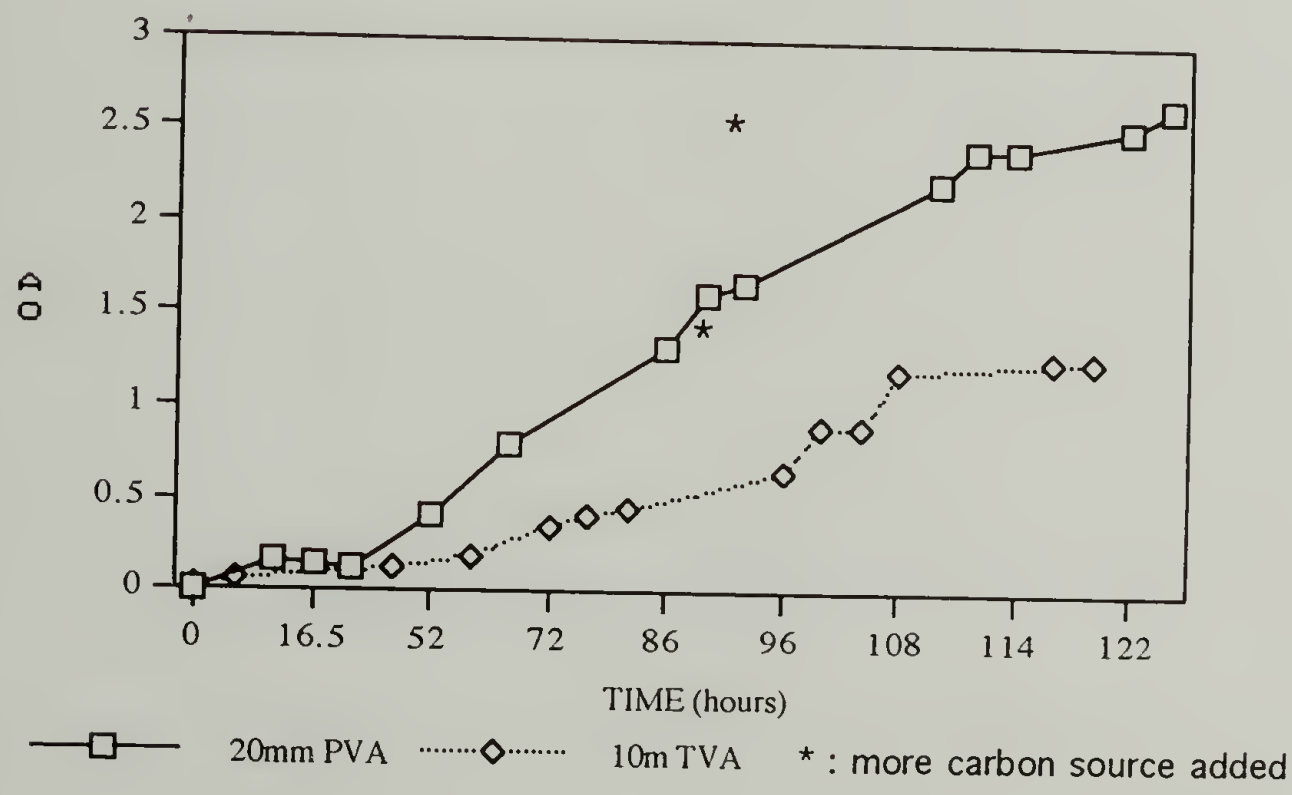


Figure 5.12 Growth curve obtained when *P. putida* was grown on PVA or TVA.

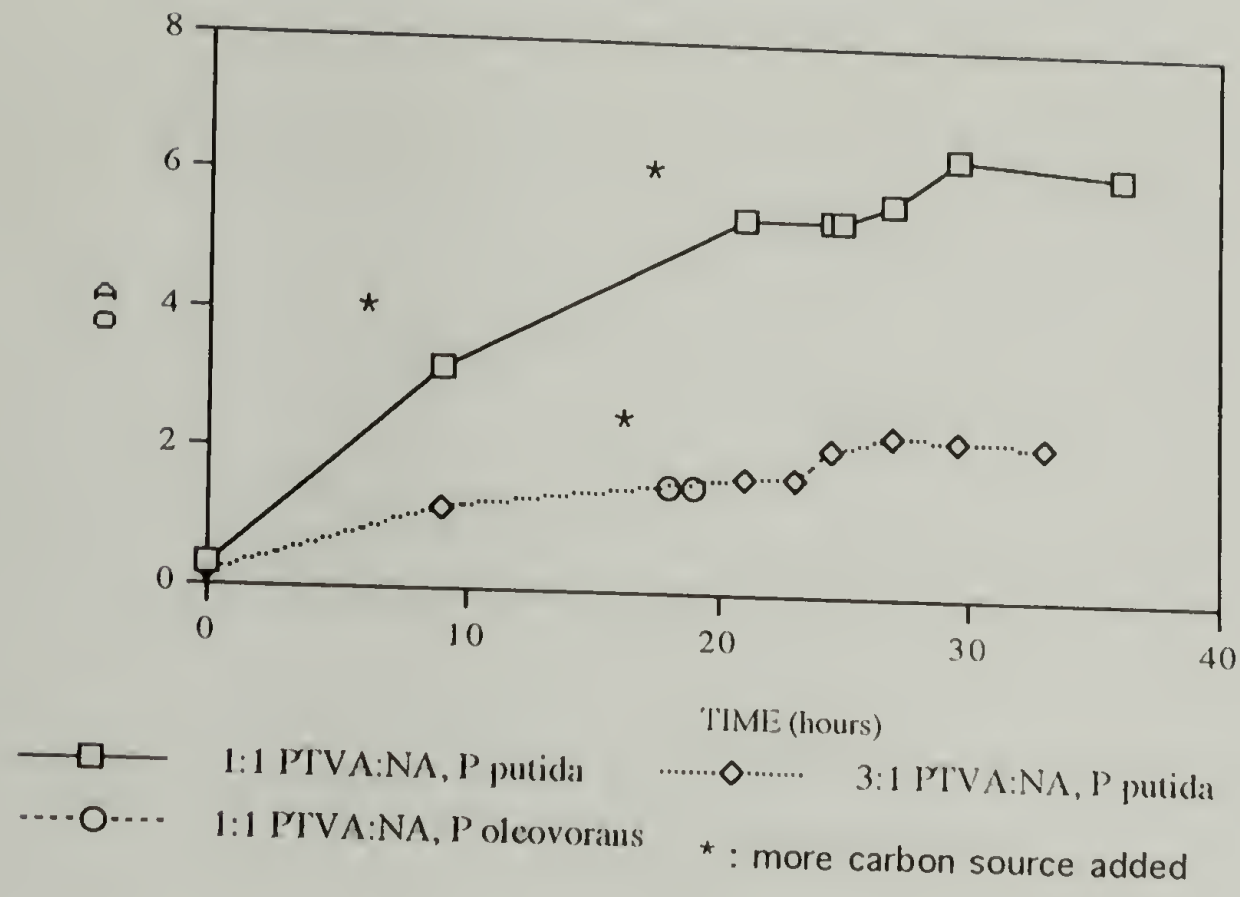


Figure 5.13 Growth curves obtained when various ratios of NA/TVA were fed to either *P. oleovorans* or *P. putida.*

Table 5.4 Growth conditions and results obtained when *P. oleovorans* and *P. putida* were fed TVA as a sole carbon source or cofed TVA with either NA or PVA

Substrates[a]	Harvest Time, hours	O.D. at Harvest	Cell Yield, g/L	Polymer Yield, g/L	Polymer Yield, %DW[b]	% Phenyl-Containing Repeat Units
P. oleovorans						
5 mM TVA 15 mM NA	21	3.4	1.4	0.21	15	3.0
5 mM TVA: 5 mM NA	19	1.5	0.68	0.05	7.0	27
5 mM TVA 5mM PVA	46	1.2	0.45	0.10	23	64 PTVA
10 mM TVA[c]	54	1.2	0.58	0.002	0.03	40
P. putida						
10 mM TVA[d]	121	1.3	0.38	0.044	12	63.4 PTVA
10 mM TVA 10 mM NA	36	6.2	2.10	0.22	11	30
15 mM TVA 5 mM NA	33	2.2	0.80	0.11	14	53

a: TVA is 5-(4'-tolyl)valeric acid, NA is nonanoic acid, TOA is 8-(4'-tolyl)octanoic acid

b: Polymer yield based on cell dry weight

c: PHN present was due to the preculture which was grown on NA

d: *P.putida* was prefed PVA as a carbon source

5.4 Conclusions

Annealing of PHPV did not result in any crystallinity. A crystalline phenyl containing polymer poly-3-hydroxy-5-(4'-tolyl)valerate (PHTV), was obtained when *P. oleovorans* was grown on 5-(4'-tolyl)valeric acid. An attempt to determine the degree of crystallinity was incomplete due to the limited amount of polymer available. Cofeeding

P. oleovorans 5-phenylvaleric acid and 5-(4'-tolyl)valeric acid resulted in a significant incorporation of the 5-(4'-tolyl)valeric acid substrate into the polymer. It was not determined whether a random copolymer or a mixture of two polymers was produced.

Growing *P. putida* on TVA either as a sole carbon source or cofeeding it with NA, resulted in a higher cell yield and polymer yield than when *P. oleovorans* was grown under the same conditions. *P. putida* proved to be superior to *P. oleovorans* in terms of its cell yield and polymer yield.

5.4 References

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CHAPTER 6

PRODUCTION OF PHA's USING PHENYLALKANES

6.1 Introduction

Pseudomonas oleovorans produces optically pure 1,2-epoxyoctane with a high degree of selectivity when grown on 1-octene [1-3]. During an experiment to increase the yield of 1,2-epoxyoctane, an intracellular storage granule, poly-3-hydroxyoctanoate (PHO), was discovered by deSmet and coworkers [4]. Apparently, *P. oleovorans* was capable of oxidizing octane to octanoic acid which could be used by the bacteria to produce poly-3-hydroxyoctanoate, PHO.

Subsequently, the amounts and compositions of the medium chain length poly-3-hydroxyalkanoates (MCL-PHA's) which were formed from *P. oleovorans* grown on alkanes of carbon chain lengths 6-12 were determined, with the results given in Table 6.1 [5]. The bacteria were able to produce MCL-PHA's when grown on these alkanes because of the presence of a catabolic OCT plasmid which encodes an alkane hydroxylase complex [5] This ω-hydroxylase system, which catalyses the following reaction sequence [1]: R-CH3 ---> RCH2-OH ---> RCHO ---> RCOOH has been extensively investigated and has been shown to exhibit broad substrate selectivity [6].

De Smet and coworkers also fed a number of phenyl-containing substrates, ethylbenzene, n-propylbenzene, n-butylbenzene and allylbenzene to *P. oleovorans* to examine the bacterias' ability to produce phenyl-containing epoxides [6]. In all cases, cell yields were low and the only epoxide detected was 1,2-epoxy-3-phenylpropane which was produced when the bacteria was grown on allylbenzene. The following factors were examined by De Smet and coworkers, in an effort to determine the reason for the lack of epoxide production:

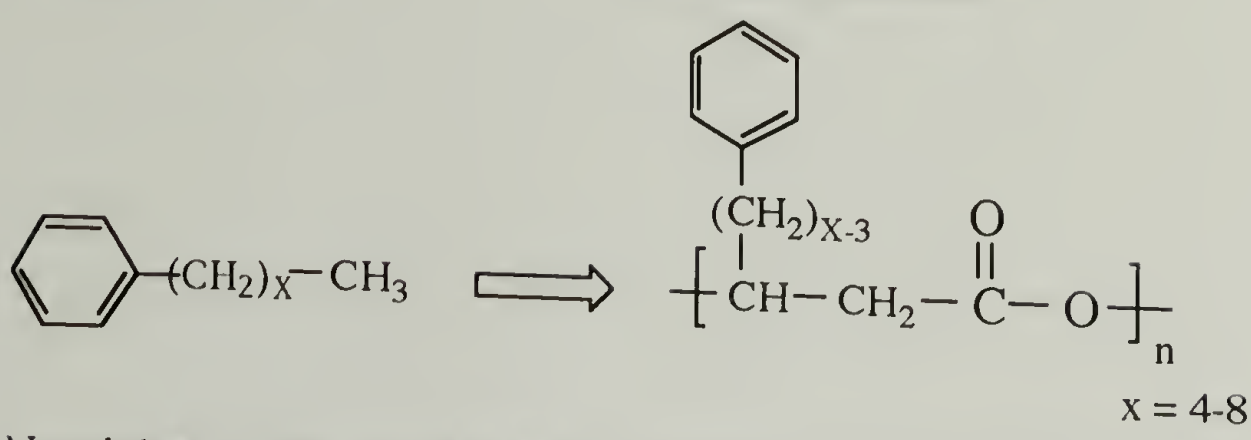
(1) The phenyl-containing substrates may be toxic to the cells. Previous studies have shown that the addition of a non-toxic compound, such as hexadecane, effectively diluted the effect of a toxic substrate by decreasing its concentration in the aqueous phase [2,7,8]. When the above mentioned phenyl-containing substrates were fed to *P. oleovorans* in the presence of 20% hexadecane, an increase in cell growth was observed, but no additional phenyl-containing epoxy compounds were produced [6]. Therefore, either these substrates were non-toxic and the lack of growth is due to another factor or the addition of 20% hexadecane did not reduce toxicity to the extent that *P. oleovorans* could support epoxide production.

(2) These phenyl-containing substrates could not induce the ω-hydroxylase system. This possibility was investigated by growing the cells on octane (which is known to induce the ω-hydroxylase) prior to the addition of the phenyl-containing substrate [6]. However, no epoxide production occurred in this case indicating that, even in the presence of a ω-hydroxylase system, *P. oleovorans* failed to metabolize phenylalkanes to form epoxides.

This section of the research dissertation describes the results obtained when *P. oleovorans* was fed commercially available phenylalkanes, and cell growth and polymer production was examined. The following phenylalkanes were used as carbon substrates, 5-phenylpentane (PPen, x=4), 6-phenylhexane (PHex, x=5), 7-phenylheptane (PHep, x=6),8-phenyloctane (POct, x=7), and 9-phenylnonane (PNon, x=8). By feeding phenylalkanes of varying chain length (C5 to C9), the optimum phenylalkyl chain length for polymer production was determined. The general structure of the phenylalkane substrates which were fed to *P. oleovorans* and the structures of the anticipated polymers are shown below:

carbon substrate

anticipated polymer



x = 4-8

Nonylphenol (NP), an inexpensive carbon source was also used as a carbon source. In addition to growing *P. oleovorans* on these phenylalkanes as a sole carbon source, these substrates were cofed with nonanoic acid, NA. In another experiment, the bacteria were grown on octane prior to the addition of the various phenylalkanes to ensure the induction of the ω-hydroxylase system.

A recent report by Casini and coworkers [9] compared polymer production when *P. oleovorans* was grown on various alkenes and diols in the presence and the absence of 20% hexadecane. Their report concluded that polymer yields were greater in cultures which were grown in a medium which contained 20% hexadecane. Because of their report of increased polymer production in the presence of hexadecane, this additive was also evaluated in the present study, and *P. oleovorans* was grown on phenylalkanes in the presence of 20% hexadecane.

Table 6.1 Aeeumulation and eomposition of PHA's obtained when *P. oleovorans* was grown on alkanes from hexane to dodecane [5].

Alkane Substrate	PHA Yield %DW[a]	PHA Composition (mol% 3HA units)						
		C6	C7	C8	C9	C10	C11	C12
hexane	2	100	---	--	---	--	---	--
heptane	11.4	---	100	---	---	--	---	--
octane	25.3	11	---	89	---	--	---	--
nonane	24.3	--	37	--	63	---	---	---
decane	21.9	10	--	66	---	24	---	---
undecane	14.3	--	23	--	63	--	---	---
dodecane	5.8	2	--	31	--	36	---	---

a : Polymer yield is based on eell dry weight

6.2 Experimental

6.2.1 Production of PHA's by *P. oleovorans* using Phenylalkanes

6.2.1.1 Phenylalkanes as the sole carbon source

Six 1L cultures of E* media containing 20 mM of PPen, PHex, PHep, POct, PNon and nonylphenol, respectively, were prepared. The growth experiment was carried out as deseribed in Seetion 2.2, with the exeeption that the phenylalkane substrates were added after autoclaving to avoid any loss of substrate.

6.2.1.2 Phenylalkanes co-fed with nonanoie aeid

Five 1L cultures of E* media which contained 10mM of various phenylalkanes and 10mM of nonanoic acid as earbon sources were prepared. The phenylalkanes used were

PHex, PHep, POct, PNon and nonylphenol. The growth experiment was carried out as described in Section 2.2 except that the phenylalkane substrates were added after autoclaving to avoid any loss of substrate.

6.2.1.3 Phenylalkanes as a sole carbon source, pre-fed with octane

Five 1L cultures of E* media which contained octane as the sole carbon source were prepared. Seventeen hours after inoculation when the O.D. of the cultures was between 0.8 and 1.5, 10mM of one of the following, PPen, PHex, PHep, PNon and nonylphenol, were added. The cultures were harvested 70 hours after the addition of the phenyl-containing substrates.

6.2.1.4 Phenylalkanes as a sole carbon source in the presence of 20% hexadecane

Four 1L cultures in which the medium consisted of 200mls of hexadecane and 800mls aqueous E* media were prepared. 20mM of PHex, PHep, PNon and nonylphenol, were used as the sole carbon source. The growth experiments was carried out as described in Section 2.2, with the exception that the phenylalkane substrates were added after autoclaving to avoid any loss of substrate.

6.2.2 Polymer Characterization

The polymer structure and composition were determined by NMR (both ^{1}H and ^{13}C), and gas chromatography, as described in Section 2.2.

6.3 Results and Discussion

6.3.1 Production of PHA's by *P. oleovorans* using Phenylalkanes

6.3.1.1 As the sole carbon source

The results obtained when phenylalkanes were the sole carbon source are summarized in Table 6.1. The bacteria grew well on both PHex and PHep, but PHep was

the only substrate on which *P. oleovorans* produced polymer. *P. oleovorans* grew poorly on PPen, PNon and nonylphenol, and these cultures were not harvested.

From the ^{1}H NMR spectrum of the polymer produced when *P. oleovorans* was grown on PHep as the sole carbon source, it was determined that the polymer contained a phenyl pendant group. ^{13}C NMR spectroscopy and gas chromatography were carried out in order to perform a more thorough elucidation of the polymer structure. The GC. chromatograph of the methyl-esters obtained by methanolysis of the polymer exhibited two peaks which were assigned as follows:

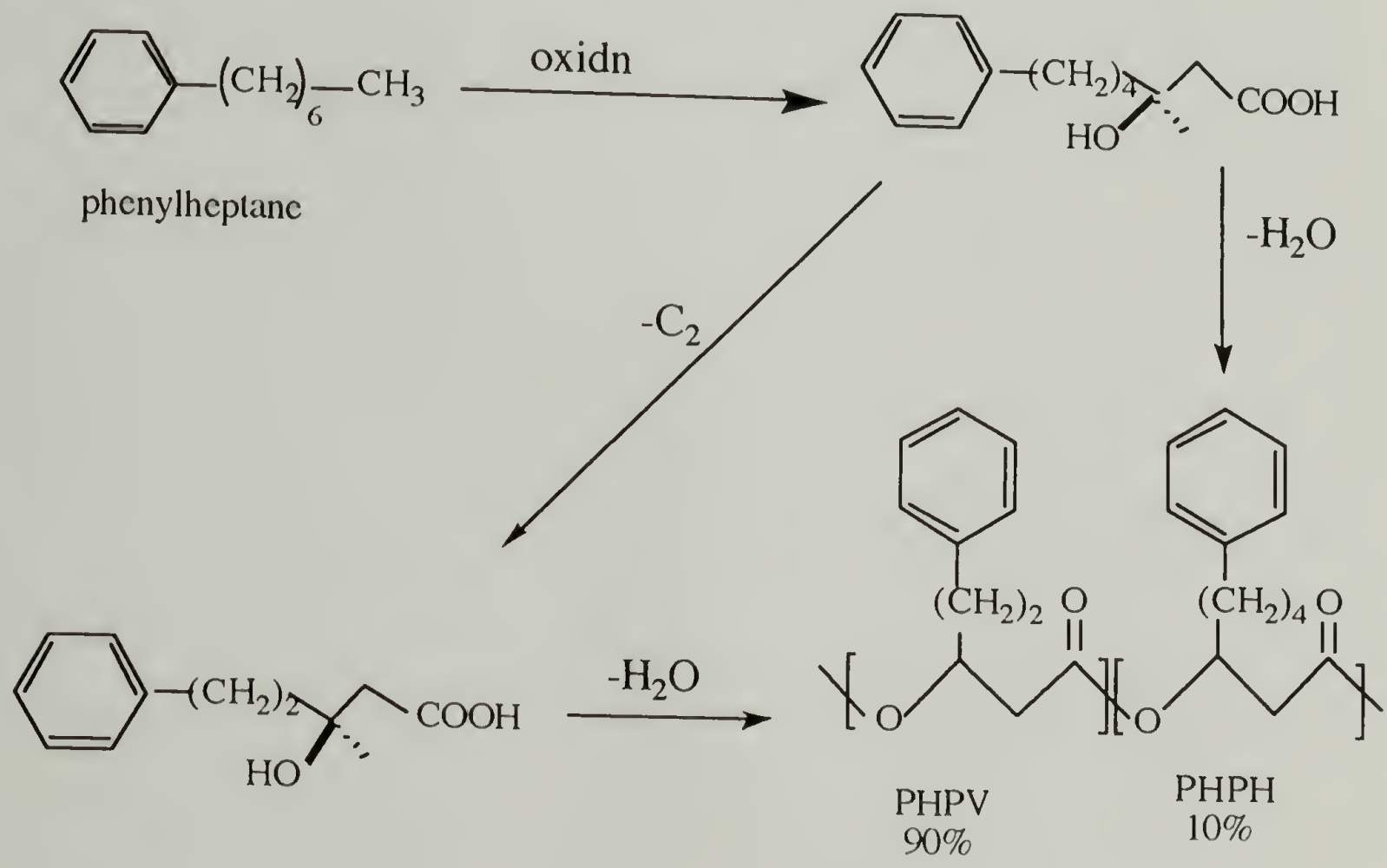
(1) The first peak with a retention time of 19.74 minutes corresponded to the methyl ester obtained from poly-3-hydroxyphenylvalerate (PHPV) polymer, and accounted for 91.1% of the total area of the chromatograph.

(2) The second peak had a retention time of 24.03 minutes. This was assigned as being the methyl ester obtained from poly-3-hydroxyphenylheptanoate (PHPH), and accounted for 8.6% of the total area of the chromatograph.

The proposed structure of this copolymer is shown in Figure 6.1, and if correct this is the first report of a microbially produced polyester with a side chain longer than 3-hydroxyphenylvalerate. The ^{13}C NMR spectrum (Figure 6.1) of the polymer confirmed the structural information obtained from G.C. analysis. That is, that the polymer obtained was composed of two repeating units: a 3-hydroxy-5-phenylvalerate and a 3-hydroxy-7-phenylheptanoate. The ^{13}C NMR spectrum of the backbone region of the polymer obtained from PHep (70-170ppm) was identical to that of PHPV, but in the side chain region (15-40ppm) there were 4 additional peaks, which are assigned to the the four methylene carbon atoms in the side chain of poly-3-hydroxyphenylheptanoate (PHPH). The chemical shift δ (in ppm) of these four carbon peaks was theoretically calculated using the following equation [10]: $\delta = -2.3 + \Sigma z_i + S$

where Σz_i is the sum of the chemical shifts of the carbon atoms which occur α, β, and γ to the carbon atom being calculated. There are three possible values for S, depending on

whether there are one, two, or three substituents on the most highly branched α carbon atom to the carbon atom being calculated. Both the zi and S values were obtained from "Struetural analysis of organic eompounds by combined applieations of spectroseopie methods" [10]. The experimental and theoretieal ^{13}C NMR ehemieal shifts for the side chain carbons of poly-3-hydroxyphenylheptanoate are eompared in Table 6.3. A reaetion scheme for the formation of the PHPV/PHPH polymer from PHep is shown below:



The predominant mechanism involves the β-oxidation of the PHep to form the 3-hydroxyphenylvalerate monomer. This result indicates that the optimum alkyl chain length for polymer production from a phenylalkane is five earbon atoms.

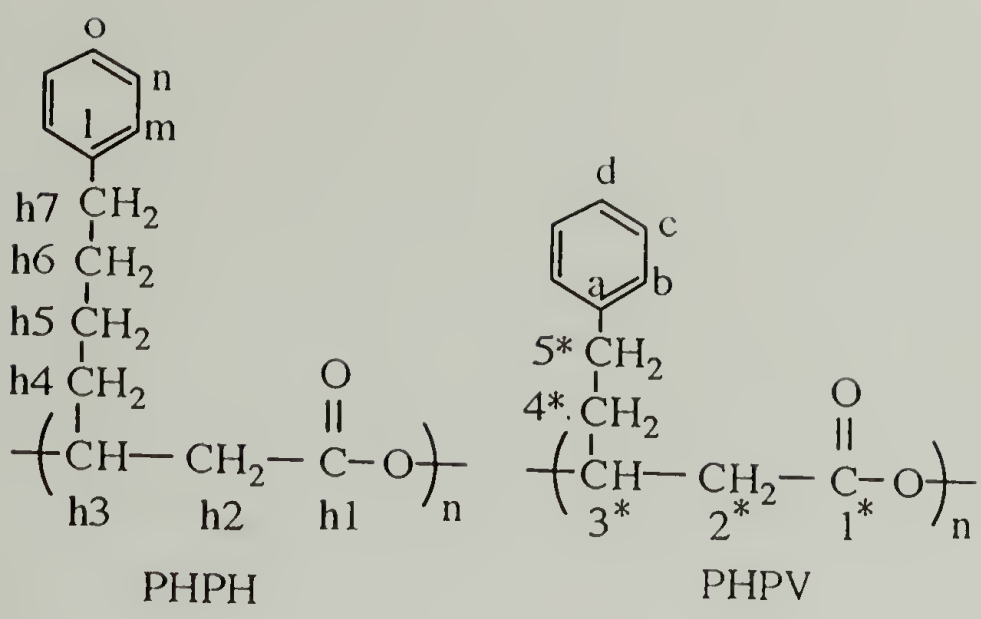
Table 6.2 Growth conditions and results obtained when *P. oleovorans* was grown on 20mM of phenylalkanes having alkylchain length of 5-9 carbon atoms, as the sole carbon source

Substrates[a]	Harvest Time, hours	O.D. at harvest	Cell Yield, g/L	Polymer Yield, g/L	Polymer Yield, %DW[b]	% Phenyl-Containing Repeating Units
PPent	----	0.4	did not harvest			
PHex	49	3.6	0.79	0.01	1.3	-
PHep	95	2.2	0.78	0.04	5.1	100
POct	----	0.2	did not harvest			
PNon	----	0.4	did not harvest			
Nonyl Phenol	----	0.3	did not harvest			

a: PPen is phenylpentane, PHex is phenylhexane, PHep is phenylheptane, POct is phenyloctane, PNon is phenylnonane,
b: Polymer yield is based on cell dry weight

Table 6.3 Experimental and theoretical chemical shifts of the side chain methylene carbon atoms from ^{13}C NMR spectroscopy (refer to Figure 6.1 for the assignment of these carbon atoms).

Side chain carbon atom	Chemical Shifts δ, ppm	
	Experimental	Theoretical
h4	36.1	36.1
h5	34.4	32.4
h6	31.4	29.2
h7	25.1	28.8



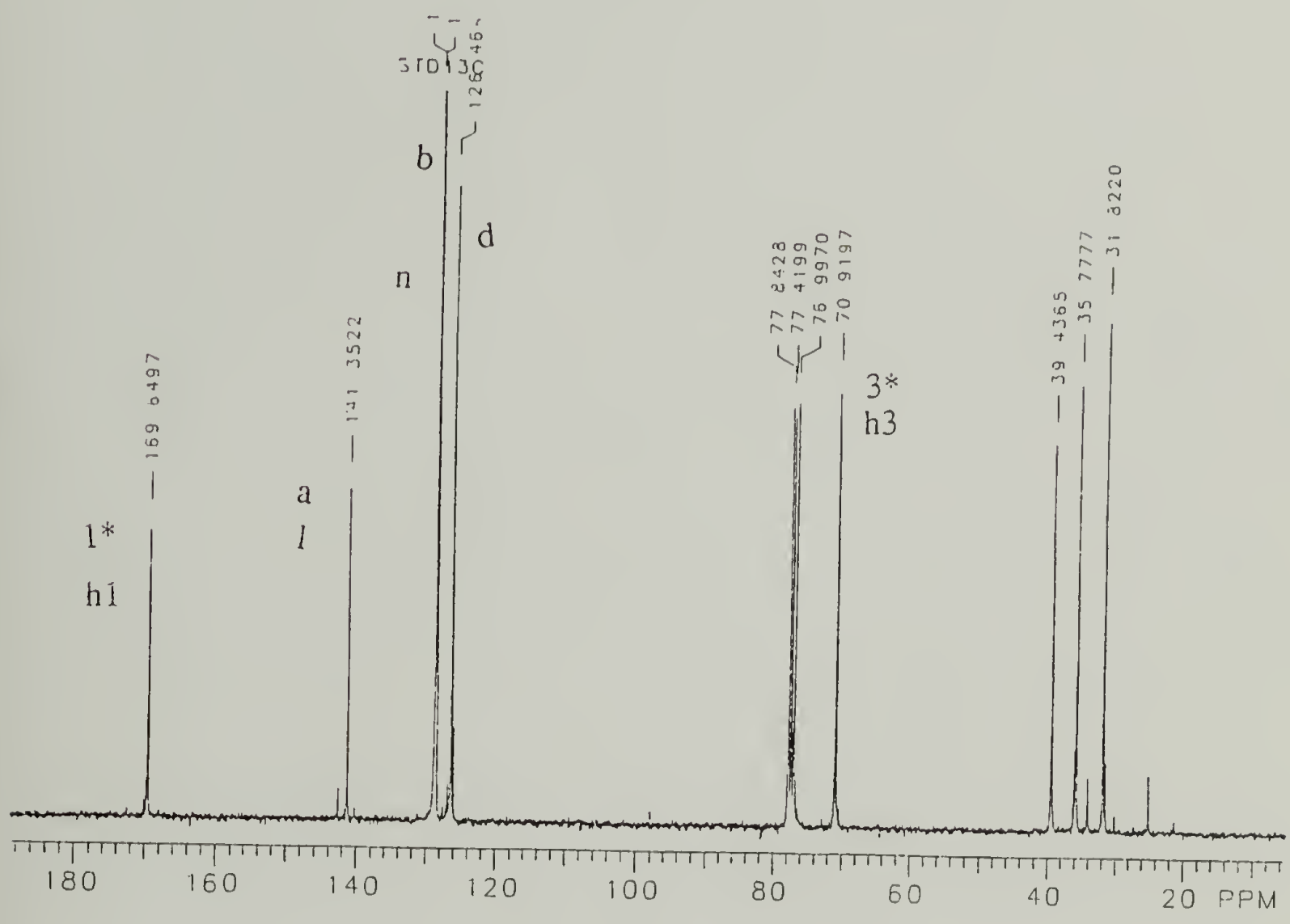


Figure 6.1 ^{13}C NMR spectrum of PHPV/PHPH, the polymer obtained when *P. oleovorans* was grown on phenylheptane as the sole carbon source.

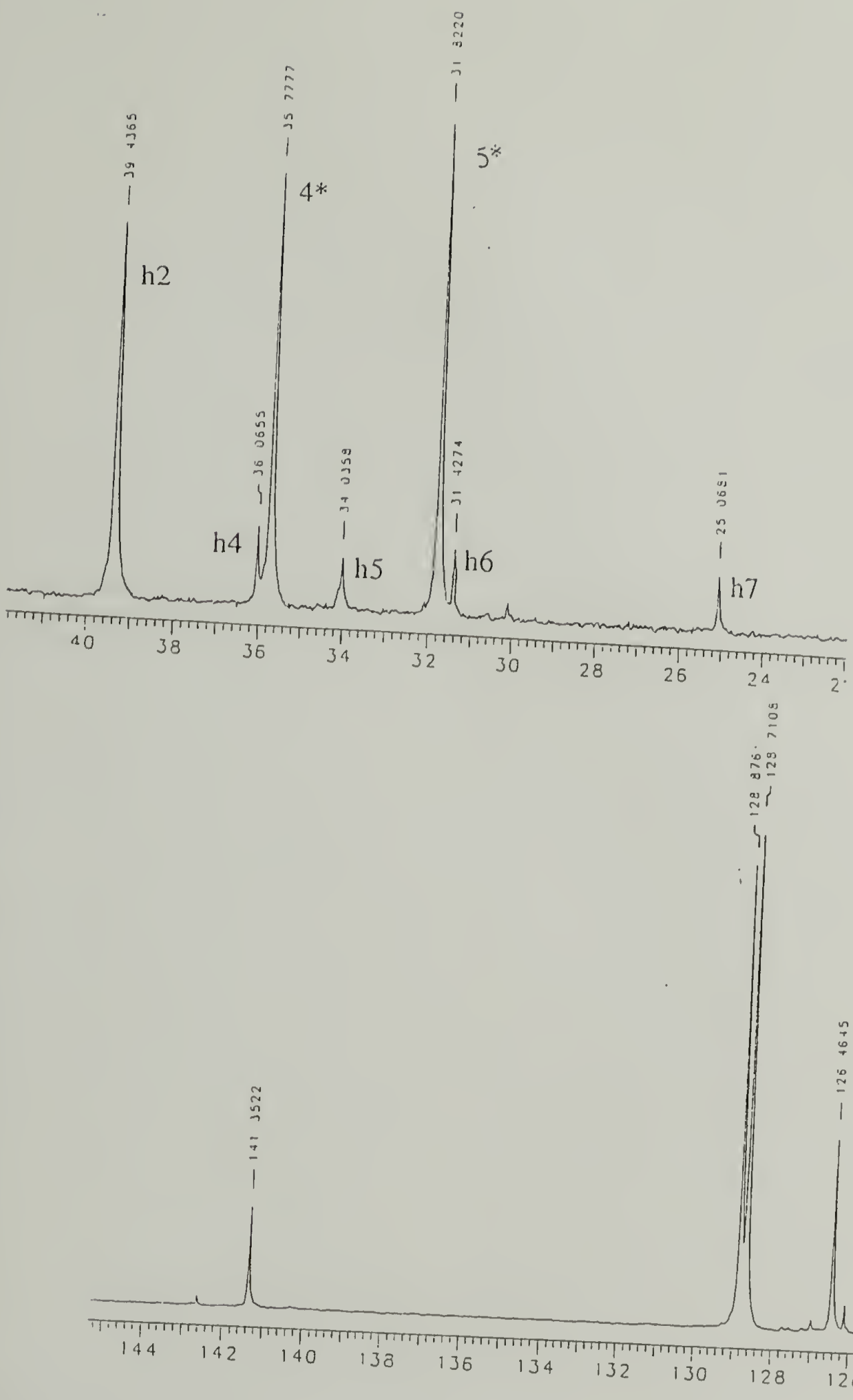


Figure 6.1 Continued

6.3.1.2.Cofeeding of phenylalkanes with nonanoic acid

The growth conditions and results when *P. oleovorans* was cofed various phenylalkanes and nonanoic acid are given in Table 6.4. Characterization of the polymer produced by NMR (both ^{1}H and ^{13}C) and methanolysis-GC, showed conclusively that the resulting polymer did not contain any repeating units having phenyl-pendant groups. The polymer produced was poly-3-hydroxynonanoate (PHN) which is the polymer produced when *P oleovorans* is grown on NA as the sole carbon source. The lack of incorporation of phenylalkane groups may be because when an alkanoic acid and an alkane were present, the alkanoic acid was consumed in preference to the alkane (which requires two oxidation steps to convert it to an alkanoic acid).

Table 6.4 Growth conditions and results obtained when *P. oleovorans* was cofed 10mM of phenylalkanes and 10mM of nonanoic acid (NA).

Substrates[a]	Harvest Time, hours	O.D. at harvest	Cell Yield, g/L	Polymer Yield, g/L	Polymer Yield, %DW[b]	% Phenyl-Containing Repeating Units
NA: PHex	23.5	3.8	1.7	0.39	24	0
NA:PHep	23.5	5.1	1.8	0.49	28	0
NA: POct	21	3.4	1.3	0.19	15	0
NA:NonylPhenol	22.5	0.4	0.9	0	0	0

a: NA is nonanoic acid, PHex is phenylhexane, PHep is phenylheptane, POct is phenyloctane

b Polymer yield is based on cell dry weight

6.3.1.3. Prefeeding *P. oleovorans* octane, prior to the addition of phenylalkanes

Octane is known to induce the ω-hydroxylase system which catalyses the conversion of alkanes to primary alcohols and finally alkanoic acids [8]. When phenylalkanes were used as the sole carbon source, the lack of polymer production may have been due to the inability of the phenyl substrates to induce this ω-hydroxylase system. Growth of *P. oleovorans* on octane prior to the introduction of phenylalkanes ensured that this enzyme system was induced. Therefore, if the absence of PHA production was due to the inability of the cells to induce the enzyme system, growing *P. oleovorans* on octane prior to the addition of phenylalkane substrates should eliminate that impediment and could facilitate production of phenyl-containing polymers.

Figure 6.2 shows the growth curves obtained when the bacteria was fed octane until an O.D. of between 0.8 and 1.3 was reached, at which point various phenylalkanes were added to the medium. A further increase in O.D. was observed when PPen, PHex or PHep were added, indicating that these substrates were being metabolized by *P. oleovorans,* but no increase in O.D. was observed when nonylphenol and PNon were fed to the *P. oleovorans* cultures. The growth conditions and results obtained are given in Table 6.5. When *P. oleovorans* was grown on octane prior to the addition of PPen, 28mg of polymer which consisted of 37% phenyl-containing polymer (as determined by ^{1}H NMR spectroscopy, Figure 6.3) was produced. The DSC thermogram of this polymer exhibited two Tg's,which corresponded to the Tg's of PHO and PHPV, respectively, indicating that a mixture of these two polymers was produced

When *P. oleovorans* was grown on octane prior to the addition of PHep, the polymer yield increased threefold compared to the yield obtained when the cells were grown on PHep from the start of the experiment (113mg versus 40mg). From ^{1}H NMR spectroscopy, it was determined that the polymer produced contained 18% alkyl repeating units and 82% phenylalkyl repeating units. The alkyl repeating units were produced from the octane prefeeding source, and the phenylalkyl repeating units were produced from

phenylheptane. The phenylalkyl units included two different types of repeating units consisted of two repeating units; approximately 90% of the repeating units were 3-hydroxy-5-phenylvalerate units and the remaining 10% were 3-hydroxyphenyl-7-heptanoate units.

When *P. oleovorans* was grown on octane prior to the addition of PHex or PNon or nonylphenol, no polymer was produced. Therefore, even in the presence of an induced ω-hydroxylase enzyme system, *P. oleovorans* did not metabolize these substrates.

6.3.1.4 PHA Production in the presence of hexadecane

The lack of polymer production with some of the phenylalkanes could also be related to the toxicity of the phenyl-containing substrates. Toxicity can be reduced by adding a non-toxic compound in which the phenyl substrates are highly miscible, thus reducing the concentration of the phenyl substrates in the aqueous phase. Hexadecane was chosen as this non-toxic second phase because it has been reported [9] that the presence of hexadecane in the E* media played a key role in the formation of polyesters by *P. oleovorans* using substrates which were incapable of supporting bacterial growth in an aqueous medium.

The substrates which were used in the study were PHex, PHep, PNon and nonylphenol. *P. oleovorans* was grown on these substrates as the sole carbon source in media which contained 20% hexadecane. The growth curve for this experiment is shown in Figure 6.4. 78 hours after inoculation, only the culture which was fed PHep exhibited growth. 10mM of PHep were added to this culture, and 10mM of NA was added to the flasks containing PNon and nonylphenol in order to determine if the cultures were still alive. The growth conditions and results for this study are given in Table 6.6. The polyester obtained from PNon, as analyzed by ^{1}H NMR and GC, was determined to be PHN, probably due to the addition of nonanoic acid after 78 hours.

Table 6.5 Growth conditions and results obtained when *P. oleovorans* was grown on octane prior to the addition of phenylalkanes.

Substrate[a]	Harvest time, hours	O.D. at Harvest	Cell Yield g/L	Polymer Yield g/L	Polymer Yield[b] %DW	% Phenyl Containing-Repeating Units
PPent	41	3.5	1.68	0.19	11	37
PHex	73	2.6	1.34	0	0	--
PHep	86	3.2	1.12	0.11	10.9	82
NonylPhenol	73	1.6	0.47	0	0	--
PNon.	73	1.6	0.34	0	0	--

a: NA is nonanoic acid, PPent is phenylpentane, PHex is phenylhexane, PHep is phenylheptane, POct is phenyloctane

b Polymer yield is based on cell dry weight

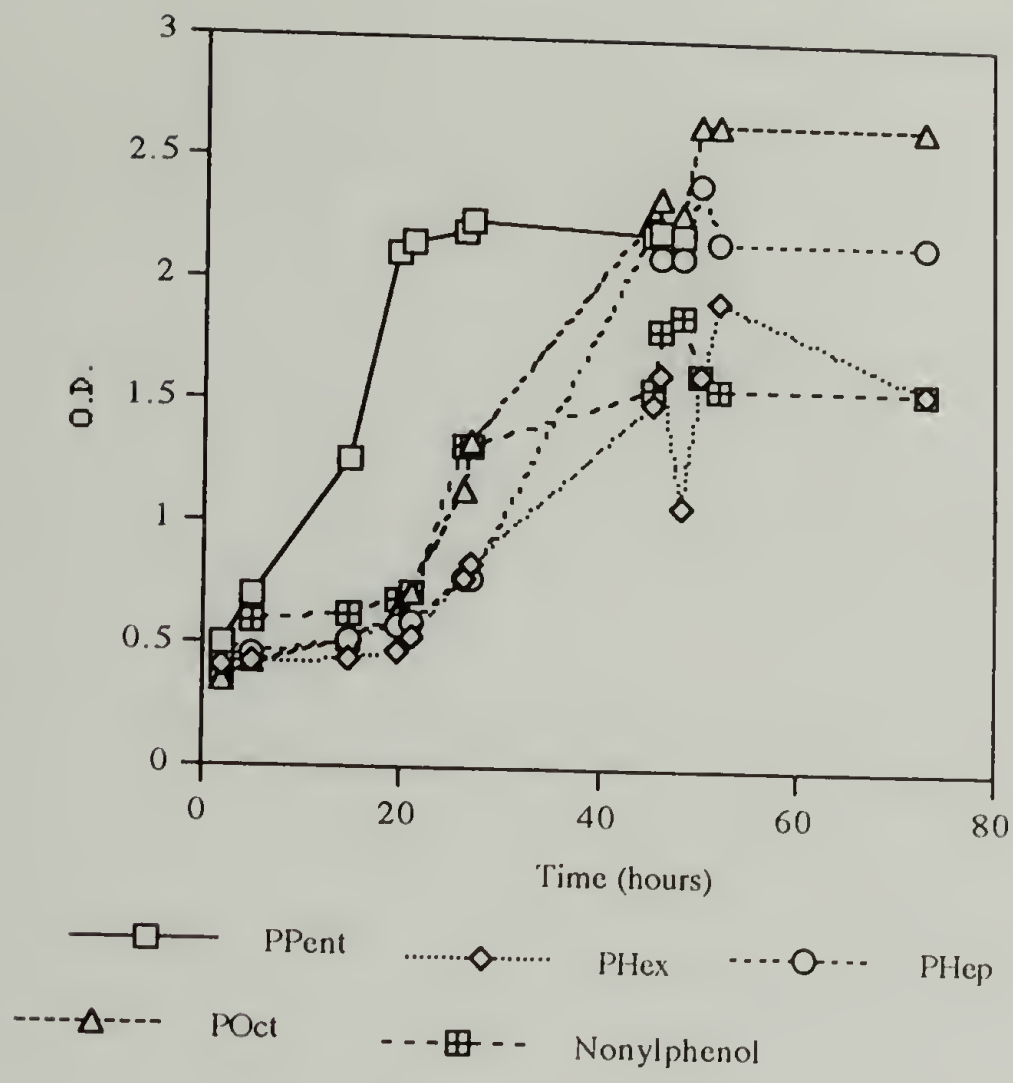
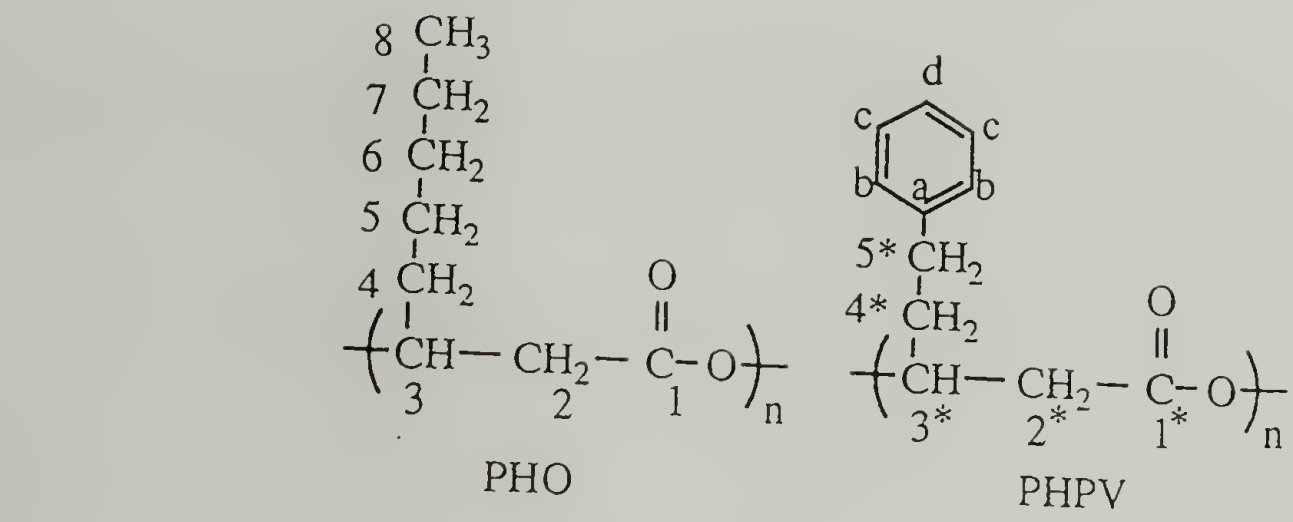


Figure 6.2 Growth curve obtained when *P. oleovorans* was grown on octane prior to the addition of various phenylalkanes



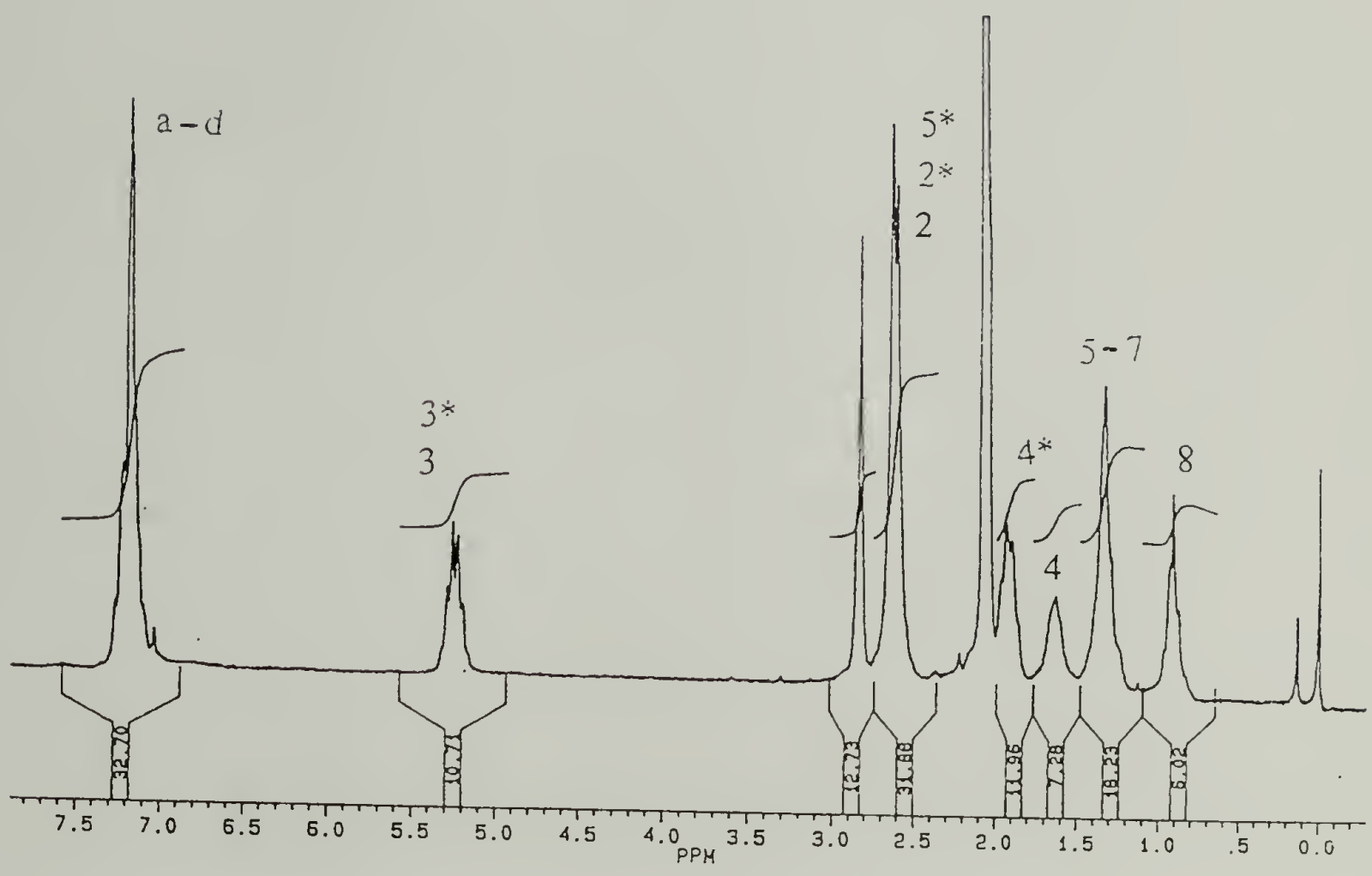


Figure 6.3 ^{1}H NMR spectrum of the mixture of poly-3-hydroxyoctanoate and poly-3-hydroxyphenylvalerate (PHPV) obtained when *P. oleovorans* was grown on octane prior to the addition of PPen.

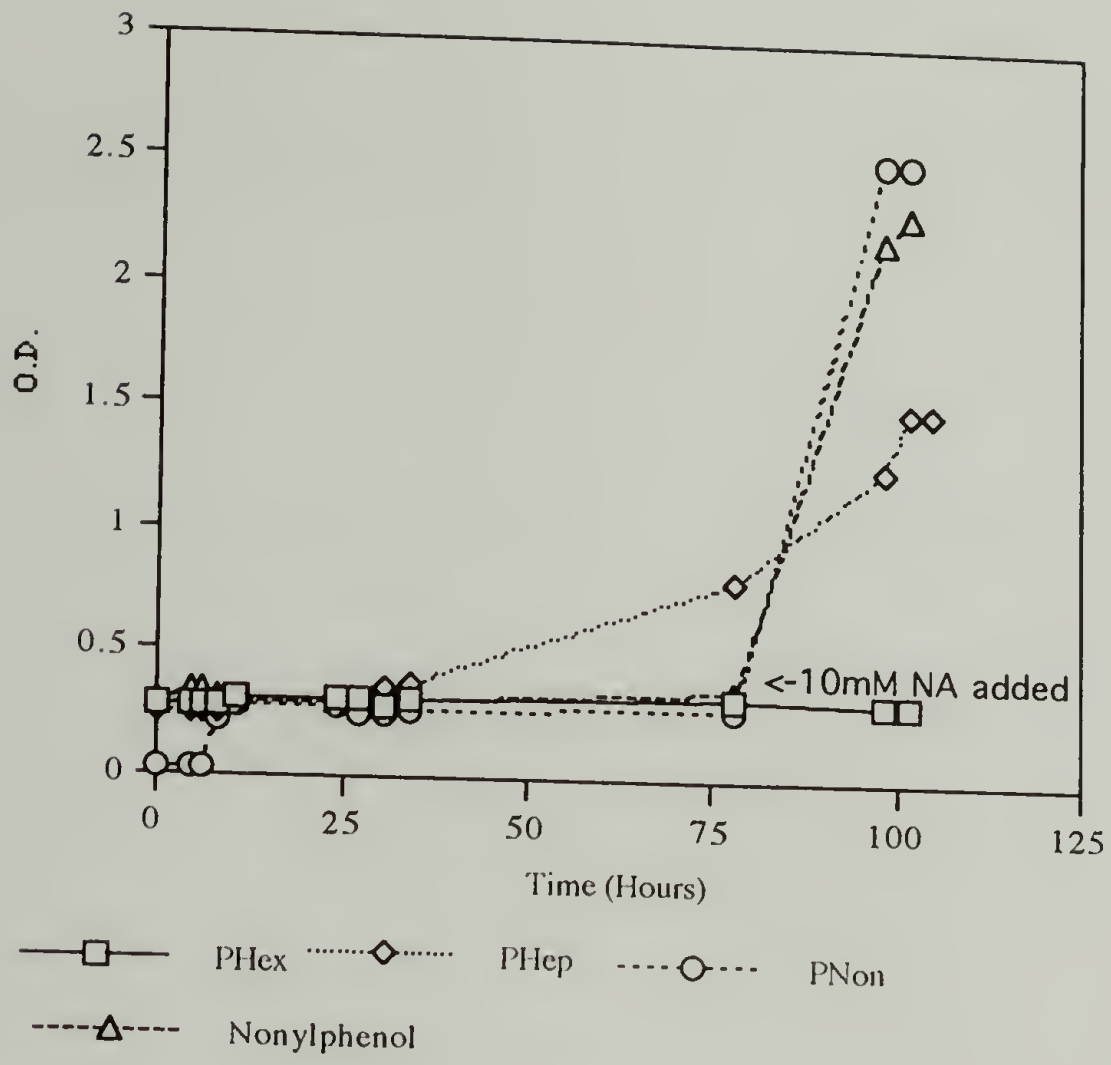


Figure 6.4 Growth curves obtained when *P. oleovorans* was grown on various phenylalkanes in the presence of 20% hexadecane

Table 6.6 Growth conditions and results obtained when *P. oleovorans* was fed 10mM phenylalkanes as the carbon source in the presence of 20% hexadecane.

Substrates[a]	Harvest Time, hours	O.D. at harvest	Cell Yield g/L	Polymer Yield, g/L	Polymer Yield, %DW[b]	% Phenyl-Containing Repeating Units
PHex	104	1.5	0.48	0.39	0	0
PHep	104	0.3	0	0.49	0.005	100
NonylPhenol	101	2.5	1.0	0.19	0	0
PNon	101	2.5	1.1	0	0.80	0

a: NA is nonanoic acid, PHex is phenylhexane, PHep is phenylheptane,PNon is phenylnonane

b Polymer yield is based on cell dry weight

c: nonanoic acid was added after 78 hours

6.4 Conclusions

Growth of *P. oleovorans* on various phenylalkanes resulted in polymer production only in the case where PHep was used. Surprisingly, the resulting polymer contained 90% poly-3-hydroxyphenylvalerate (PHPV) and 10% poly-3-hydroxyphenylheptanoate (PHPH) units, indicating that the optimum alkyl chain length for polymer production was five carbon atoms. This observation appears to be the first report of a microbially produced phenyl-containing polyester with a 3-hydroxy-7-phenylheptanoate repeating unit.

When *P. oleovorans* was cofed an equimolar mixture of NA and various phenylalkanes, the sole polymer produced was PHN. The lack of incorporation of the phenylalkanes into the polymer backbone may have been because when an alkanoic acid and an alkane were present, the alkanoic acid may be consumed in preference to the alkane (which requires two oxidation steps to convert it to an alkanoic acid).

Growth of *P. oleovorans* on octane prior to the addition of various phenylalkanes, (in order to induce the ω-hydroxylase enzyme system) resulted in polymer production when either PPen and PHep was used. In both cases the predominant phenylalkyl repeating unit was 3-hydroxyphenylvalerate, indicating, once again, that the optimum alkyl chain length when a phenylalkyl substrate was used was 5 carbon atoms.

When the media was altered so that it consisted of 20% hexadecane and 80% aqueous E* media, in order to dilute any toxic effect of the phenylalkane substrates, no increase in the amount of incorporation of the phenyl-containing repeating units into the polymer backbone was observed. Therefore, either these substrates were not toxic and the absence of growth was due to another factor, or the addition of 20% hexadecane did not reduce toxicity to the extent that *P. oleovorans* was able to support polymer production.

6.5 References

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CHAPTER 7

DEGRADATION OF FUNCTIONAL PHA's

7.1 Introduction

Extracellular degradation of poly-3-hydroxyalkanoates (PHA's) can occur when a microorganism secretes PHA specific depolymerases, which can hydrolyze the polymer to its corresponding 3-hydroxyalkanoates and oligomeric esters. These water-soluble degradation products can be ingested by the bacteria and utilized as nutrients. Most of the research on the extracellular degradation of PHA's has concentrated on short-chain-length PHA's (SCL-PHA's). Aerobic and anaerobic PHB-degrading bacteria have been isolated in various environments in which they can excrete one or more extracellular depolymerases. These environments include soil, (from which *Pseudomonas lemoigne* [1] was isolated), molds [2], activated sludge (from which *Alcaligenes faecalis T1* [3,4] was isolated) and sea water (from which *Comomonas testeroni* [5] was isolated). The extracellular depolymerase of *P. lemoigne* [6], and *A. faecalis* [3] have been isolated and characterized.

The degradation rates of SCL-PHA's are strongly dependant on copolymer composition and morphology. Kunioka and coworkers [3,7] compared the rates of degradation of PHB, a copolymer of 91% 3HB and 9% 4HB, and a copolymer of 50% 3HB and 50% 3HV in soil. The degradation rates of these polymer decreased in the following order: P(3HB-co-4HB) > P(3HB) > P(3HB-co-3HV). Amorphous sections of SCL-PHA's degrade faster than crystalline sections [8-10], so if the hydrolyse enzymes involved in these degradation processes were isolated, because of their lower degree of crystallinity, medium-chain-length PHA's (MCL-PHA's) should degrade readily.

While the extracellular degradation of PHB and PHBV has been studied intensively there have been few reports regarding the extracellular degradation of other PHA's.

However, Schirmer and coworkers [11] recently discovered 26 bacteria capable of growing on and degrading poly-3-hydroxyoctanoate (PHO). This report contained the first real proof that extracellular degradation of MCL-PHA's occurs. The extracellular depolymerase from one of the most efficient strains, which was identified as *Pseudomonas fluorescent* GK13, was isolated and characterized, and even more recently, it was reported that crosslinked poly-3-hydroxyoctanoate-co-undecenoate was degraded using the extracellular depolymerase from *P. fluorescent* GK13 [12].

It is known that PHA-producing bacteria can be divided into two classes, one of which produces SCL-PHA's and the other produces MCL-PHA's, and the polymerase enzymes which produce each of these types of PHA's have been isolated and characterized. SCL-PHA's are produced by different enzymes than those one which produce MCL-PHA's. In that regard, it is interesting to note, too, that the extracellular depolymerase enzyme from *P. fluorescent* GK13, which degraded MCL-PHA's did not degrade the SCL-PHA's, PHB or PHPV. Therefore, it appears that in addition to the existence of two separate polymerase systems, there exists two separate extracellular depolymerase systems, one which degrades SCL-PHA's and another which degrades MCL-PHA's.

7.1.1 Intracellular Degradation

Intracellular degradation is the degradation of an intracellular storage polymer by a depolymerase enzyme produced by the same cell which synthesizes the polymer, and which is active inside the cell. An earlier study carried out in this laboratory [14] showed that the intracellular degradation of PHN occurred rapidly once the substrate concentration fell below 0.5mM.

The first experiment conducted in this section of the dissertation research program was an investigation of the bacteria's ability to degrade its stored polymer. The samples studied were cell of *P. oleovorans* which contained either poly-3-hydroxynonanoate (PHN), poly-3-hydroxyphenylvalerate (PHPV) or a blend of PHN/PHPV. The immiscible

blend of PHN/PHPV was produced when *P. oleovorans* was cofed an equimolar mixture of nonanoic acid (NA) and 5-phenylvaleric acid (PVA). In that case, analysis of the medium by gas chromatography showed that the bacteria consumed 60% of the NA before it began to consume the PVA [14]. In chapter 3 of this dissertation the intracellular location of the two polymers in this blend was investigated. A transmission electron micrograph of *P. oleovorans* which was grown on a mixture of NA/PVA, and contained a blend of PHN/PHPV (Figure 3.6) showed that both polymers occurred in the same granule. PHN polymer occurred in the core of the granule and PHPV polymer accumulated around this core.

This section of the dissertation research program describes the ^{13}C NMR spectroscopy experiments which were carried out on cells which contained granules of either PHN, PHPV or a blend of both PHN/PHPV, to monitor the intracellular degradation of each polymer, and to determine if the presence of the PHPV in the outer shell of the granule affected the intracellular degradation rate of PHN in the core of the granule. As the rate of production of PHPV is slower than that of PHN, it was anticipated that the intracellular degradation of PHPV would also occur at a slower rate. ^{13}C NMR spectroscopy proved to be an ideal technique for monitoring intracellular degradation, because the sample preparation involved was minimal and the technique was non-destructive towards the cells even at a temperature of 345K. It has been shown that the cells are still viable , after prolonged periods at temperatures at 318K. This technique has also been used to monitor the *in vivo* degradation of PHA's in cells of *Acaligenes eutrophus,* which contained PHB, and in cells of *P. oleovorans,* which contained PHO [15].

The morphology of the *in vivo* polymer has been the subject of much debate [16]. The results of the initial carbon replica electron microscopy [17] and electron microscopy experiments [18] carried out in the 1960's concluded that the polymer *in vivo* was crystalline. However, in the 1980's with the advent of new characterization techniques, including ^{13}C NMR spectroscopy [19] and wide-angle X-Ray scattering (WAXS) [20,21],

it was proven that the polymer *in vivo* was amorphous. Another reason for the discrepancies between the results of the experiments carried out in the 1960's and those carried out in the 1980's was that the earlier experiments were performed on freeze dried cells. It has since been shown that although the polymer in freeze dried cells is still amorphous, its mobility is severely hampered [22].

The two principal theories, which have been suggested to explain why the polymer is amorphous *in vivo* but becomes crystalline upon extraction are the following:

(1) the granules consist of polymer, water, lipid and protein, and that the combination of water and lipid act as a plasticizer keeping the granule in the amorphous state [22,23].

(2) the PHB does not crystallize *in vivo* because of the slow nucleation process which occurs in the small granules of highly pure polymer. To investigate the latter suggestion, Horowitz and coworkers [24] have taken crystalline PHB and sucessfully reverted it back to the amorphous granule morphology using a surfactant to stabilize the granules.

7.1.2 Extracellular Degradation

In previous extracellular degradation studies on poly-3-hydroxyoctanoate (PHO) carried out in this laboratory, films of PHO were placed in leaf compost, sewage sludge, an enrichment culture and a fungal culture. When the films were placed in compost , they melted, and were not recovered. When the films were placed in the other environments described above, no degradation was observed [25]. In this extracellular degradation study carried out on poly-3-hydroxynonanoate (PHN), films of PHN were cast from 20% solutions of chloroform and sent to Dr. Alan White at Eastman Chemical Co. in Kingsport, Tennessee, where the films were evaluated in a bench scale composting unit which is currently being used to monitor the degradation of cellulose esters [26].

Extracellular degradation studies on both PHN and PHPV were carried out in our laboratory, using a 'clear zone ' technique and an extracellular depolymerase enzyme secreted by *Pseudomonas maculicola* [27].

7.2 Experimental

7.2.1 Intracellular Degradation of PHN, PHPV and a blend of PHN/PHPV

P. oleovorans was grown on the following substrates:

NA for the production of PHN as an intracellular storage granule

PVA for the production of PHPV as an intracellular storage granule

an equimolar mixture of NA/PVA, which resulted in the production of granules containing a physical blend of PHN and PHPV

The cultures were grown in E* media in a 12L fermentor containing the relevant carbon substrate(s). When *P. oleovorans* was grown on NA or on an equimolar mixture of NA/PVA, the cultures were harvested when the maximum O.D. was achieved. The culture was centrifuged at 4000rpm for 20 minutes, and the cells from 9L of media were resuspended in 9L of E* media which contained no carbon source, so that the bacteria were forced to consume their stored polymer in order to survive. The remaining 3 L were harvested at time zero, the time which corresponded to the transfer of the cells to the media which contained no carbon source. These three samples were used to determine the experimental error involved. Periodically over a 100 hour period, 1L of the remaining 9L culture was removed from the fermentor, and split into two 500ml aliquots, which were centrifuged at 4000rpm for 20 minutes. The cells from one aliquot were examined by ^{13}C NMR spectroscopy. The cells in the other aliquot were lyopholized, the polymer was extracted, and the cell and polymer yields (based on cell dry weight) were determined. In the case where a blend of PHN/PHPV was produced, the polymer composition was determined using ^{1}H NMR spectroscopy.

The experiment was modified in the case where the cells were grown on PVA as the sole carbon source. 12L of culture was grown as outlined in Section 2.2. However, due to low cell and polymer yields, it was necessary to use the cells from two liters of culture for the NMR spectroscopy studies. Periodically, over a 100 hour period, 2L aliquots were removed and centrifuged at 4000rpm for 20 minutes. After the cells were analyzed by ^{13}C NMR spectroscopy, they were lyopholized and extracted to determine the cell and polymer yields and the percent polymer (based on cell dry weight).

^{13}C NMR spectroscopy was used to monitor the intracellular degradation of PHN, PHPV and a blend of PHN/PHPV within cells of *P. oleovorans* . The analysis was carried out at Mount Holyoke College under the direction of Prof. Sheila Browne. The NMR spectra were obtained on an IBM 270MHz spectrometer, and the temperature of the probe was calibrated with a standard glycerol sample. Chemical shifts were referenced against 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The samples were prepared as described below:

Each 500ml aliquot was centrifuged at 4000rpm for 20 minutes. The supernate was discarded, deuterium oxide (D_2O) was added to bring the total volume to 6mls and 2.5mls of the sample was transferred to a ^{13}C NMR tube. A coaxial insert containing the standard, DSS was placed in this tube. In the case of the cells containing PHN and a blend of PHN/PHPV, 25000 scans were obtained over a 24 hour period at 318K. As intracellular PHPV was less mobile than the PHN, it was necessary to use a temperature of 345K to obtain a sharp ^{13}C NMR spectrum of this polymer.

7.2.2 Extracellular Degradation Studies

7.2.2.1 Extracellular degradation on PHN in a bench composting unit

Solution cast films of PHN were sent to Dr. Alan White at the Eastman Chemical Company, where they were cut into strips of the following dimensions: length 15.2cm,

width 1.3cm, and thickness 0.5mm. They were placed in a bench scale compost environment, in which the compost was composed of the following:
3.5kg of dehydrated alfalfa meal, 1.3 Kg of cottonseed meal, 1.4Kg of poplar sawdust, 1.0Kg of fresh cow manure, 1.5Kg of finely shredded newspaper, 480g of $CaCo_3$, 40g of $NaHCO_3$, and 13L of water. These substituents were added to a Hobart Mixer and blended to obtain particles of 3-4mm. The carbon-to-nitrogen ratio of the starting compost was 30:1 and the initial pH was 7.2. The temperature inside the compost can reach 70°C in the initial phase of composting, known as the thermophilic phase. Because this temperature is above the melting temperature of the PHN, the polymer films was placed in the composting unit only after the thermophilic phase. After 30 days the films were removed, washed in a neutral detergent for one hour, dried in a vaccum for 3 days, and weighed to determine weight loss.

7.2.2.2 Extracellular Degradation of PHN and PHPV using the clear zone technique

The clear zone experiment was carried out as follows; 10mls of modified E* media (with or without carbon source) and 2.5% agar were placed in a petri dish as the bottom layer, and on top of this layer, after solidification of the bottom layer was placed a solution of 6mls of a colloidal suspension of polymer of known concentration and 30mls of a 2.5 % agar solution. The colloidal suspension was made according to the method of Ramsey and coworkers [28]. The top layer in the petri dish was innoculated using 5µl of *Pseudomonas maculicola* grown on 20mM sodium octanoate in E* media, and the contents of the petri dish were examined periodically by measuring the area of the clear zone formed (if any). The amount of polymer degradation which occurred could be calculated using the following equation:

$$\text{Polymer weight loss} = \frac{(\text{Clear zone area} \times \text{Total weight of polymer top layer})}{\text{Total area of petri dish}}$$

7.3 Results and Discussion

7.3.1 Intracellular Degradation of PHN, PHPV and a blend of PHN/PHPV

^{13}C NMR spectroscopy proved to be a powerful tool for monitoring the intracellular degradation of bacterial polyesters. Figure 7.1 shows the ^{13}C NMR spectrum of the standard sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), which exhibited four sharp peaks. Figure 7.2 shows the ^{13}C NMR spectra of cells of *P. oleovorans* which contained PHN, with all of the peaks assigned. The carbonyl peak at 170 ppm is relatively sharp, the other two backbone peaks, the -CH- peak at 74ppm and the $-CH_2-$ peak at 47 ppm, are broader than the carbonyl peak and are also broader than the sidechain peaks. Figure 7.3 shows the ^{13}C NMR spectra of cells which contained PHN taken at three different temperatures, 298, 310, and 318K. The spectrum obtained at 318K shows the sharpest peaks and the highest signal-to-noise ratio. For this reason the ^{13}C NMR spectra of cells containing PHN and a blend of PHN/PHPV were obtained at 318K. It was observed that increasing the temperature did not have such a profound influence on the side chain peaks, which indicated that the side chain peaks have similar mobility at the lower and higher temperatures in the temperature range used.

Figure 7.4 shows the ^{13}C NMR spectra of three samples of cells (1A, 1B, and 1C) which contained PHN as the sole intracellular storage polymer. These three samples were harvested at time zero, which was the time when the culture was centrifuged and resuspended in carbon-free medium, in order to determine the amount of error involved in this spectroscopic technique. Figure 7.5 shows the ^{13}C NMR spectra of samples 2-8, which were harvested periodically over a 100 hour period, after the culture was resuspended in a medium which contained no carbon source. The height of the carbonyl peak at 170 ppm, C=O/DSS, was compared to the height of the DSS standard peak at 0 ppm in order to monitor the intracellular degradation of PHN. As was mentioned previously, each 1L sample was divided into two 500ml aliquots. ^{13}C NMR spectroscopy was used to analyse one of the 500ml aliquots, while the other 500ml aliquot was

centrifuged and the polymer extracted to determine cell and polymer yields and the percent polymer (based on cell dry wcight). The ratio of the carbonyl peak to the standard peak, C=O/DSS, the corresponding cell yield, polymer yield, the initial percent polymer (based on cell dry weight) and the percent polymer remaining when PHN was the sole intracellular storagc granule are given in Table 7.1. Figure 7.6 is a comparison of the two methods used to monitor intracellular degradation. Plots of the polymer remaining (determined by extracting onc 500ml aliquot) and C=O/DSS (determined by performing ^{13}CNMR on the other 500ml aliquot) versus harvest time are shown in Figure 7.6. The two plots in Figure 7.6 overlay each othcr closely, showing that ^{13}C NMR spectroscopy is an excellent techniquc for monitoring the intracellular degradation of PHN.

Figure 7.7 contains the ^{13}C NMR spectrum of the cells which contained PHPV as the solc intracellular storage granule. In this case the height of the phenyl peak at 128ppm was compared to the height of the DSS standard peak at 0 ppm (AR/DSS) to monitor the intracellular degradation of PHPV. Figure 7.8 shows the ^{13}C NMR spectra of samples 1-6, which are cells that contained PHPV as the sole intracellular polymer that were harvested over a 115 hour period, after the culture was resuspended in a carbon-free medium. The ratio of the phenyl peak to the standard peak, AR/DSS, the corresponding cell yield, polymer yield and the initial percent polymer (based on cell dry weight) and the percent polymer remaining are given in Tablc 7.2. Plots of the amount of polymer remaining (g/L) and AR/DSS ratio versus harvest time when PHPV was the sole intracellular polymer are shown in Figure 7.9. In this case the two plots of the amount of polymer remaining and AR/DSS ratio versus harvest time do not overlay each other as closely as the corresponding plots when PHN was the sole intracellular storage polymer. This result may be due to the fact that PHPV is not as mobile as PHN, resulting in lower signal-to-noise ratios and less sharp ^{13}C NMR spectra.

Regardless of the method used to monitor degradation, it appeared that at the time that thc cells were transferred to the carbon-free medium (time= 0 hours), the polymer

production had not yet reached a maximum. The maximum polymer yield was obtained from sample 3 which was harvested 40 hours after the culture was resuspended in a carbon-free medium. This result corresponds with the results of Fritzsche and coworkers [29], who showed that PHPV production reached a maximum 24 hours after the maximum O.D. was achieved. The intracellular PHPV did not appear to degrade until after sample 5 was harvested at 90 hours. At 115 hours after the cells were resuspended in medium that contained no carbon source, both methods indicated that degradation had occurred. It appears, therefore, that in addition to producing PHPV at a much slower rate, *P. oleovorans* also degraded PHPV at a much slower rate than it degraded PHN.

In this case, where there were two types of intracellular storage polymers present, the two ratios, C=O/DSS and AR/DSS, were calculated in the manner in which they would be calculated if each polymer were the sole storage polymer present. Figure 7.10 shows the spectra of the three samples 1A, 1B, and 1C which were harvested at time zero. The C=O/DSS and AR=DSS ratios calculated showed the results obtained by analysis by ^{13}C NMR spectroscopy were highly reproducible. Figure 7.11 shows the ^{13}C NMR spectra of the samples 1-5, which are cells of *P. oleovorans* which contained both PHN and PHPV, harvested periodically over a 100 hour period, after the cells were resuspended in a culture which contained no carbon source. C=O/DSS, AR/DSS, the corresponding cell yield, polymer yield, and percent polymer present (based on cell dry weight) and the percent polymer remaining, are given in Table 7.3. Table 7.4 gives the polymer yield (g/L) and the polymer composition (as determined by ^{1}H NMR spectroscopy) for each sample harvested over a 100 hour period.

Figure 7.12 is a plot of the PHN polymer remaining (g/L) and C=O/DSS versus harvest time when both PHN and PHPV were present in the cell. Figure 7.13 is a plot of the PHPV polymer remaining (g/L) and D=AR/DSS versus harvest time when both PHN and PHPV were present in the cell. From Figure 7.12 and 7.13 it can be seen that while both polymers degraded *in vivo*, PHN degraded faster than PHPV. In the case where there

were two intracellular polymers present, the plots of polymer remaining (g/L) versus harvest time and the plot of C=O/DSS or AR/DSS versus harvest time (Figure 7.12 and 7.13) do not overlay each other as closely as the corresponding plots when PHN was the sole intracellular polymer present. This result may have been obtained because, in the case where PHN was the sole intracellular storage polymer, the amount of polymer remaining was determined by weighing the extracted PHN, whereas in the case where a blend of two polymers was obtained, the amount of each type of polymer present was calculated as the product of the fraction of each polymer present (as determined by ^{1}H NMR spectroscopy) and the weight of the polymer blend. Therefore, in the case of the intracellular degradation of the PHPV, there was an additional experimental error involved due to the use of 1H NMR spectroscopy.

In Figure 7.14 the rate of degradation of the PHN, when it was present in the blend, was compared to the rate of degradation when it was the sole intracellular storage polymer. From Figure 7.14 it can be seen that the rate of degradation of PHN was not significantly affected by the presence of the PHPV. That is, PHN degraded at the same rate when it was the sole polymer present *in vivo* as when it was present as a component of a blend of PHN/PHPV. The rates of degradation of PHPV, when it was the sole intracellular polyester and when it is present as a component of a blend, are shown in Figure 7.15. When PHPV was present as a component of a blend, it degrades faster then when PHPV was the sole intracellular storage polymer. It is possible that PHN and PHPV were degraded by the same intracellular depolymerase which operated more efficiently in the presence of PHN.

7.3.2 Extracellular Degradation

7.3.2.1 Degradation of PHN using a bench scale composting unit

This experiment was carried out at the Eastman Chemical Company, Kingsport Tennessee, under the direction of Dr. Alan White. The average weight loss observed in 10

PHN samples was 5%. The fact that there was a reproducible weight loss of 5% and a change in the surface morphology (the surface of the films was pitted) indicated that the samples were beginning to degrade and 30 days may not have been a sufficient time frame for substantial degradation to occur. PHB and a copolymer of 78% 3HB and 22% 3HV placed in the same environment lost 75% and 100% respectively, of their total weights after 30 days. Brandl and coworkers [30] suggested that the extracellular degradation of MCL-PHA's might be expected to take longer than that of SCL-PHA due to the more hydrophobic nature of the MCL-PHA's.

7.3.2.2 Extracellular degradation of PHN and PHPV using the clear zone method

The extracellular degradation studies were carried out under the direction of Dr L. J. Foster. The presence of a clear zone around the bacterial colony showed that polymer present in the colloidal suspension of the agar was degraded by an extracellular depolymerase excreted by *P. maculicola*. While clearing occurred when a colloidal suspension of PHN was used and 29% of the polymer was degraded, no clearing occurred when PHPV was used. Polymer composition obviously has a profound effect on the extracellular degradation .

Table 7.1 Harvest time and the results obtained in the intracellular degradation study of PHN.

Sample Number	Harvest Time, hours	Cell Yield, g/L	Polymer Yield, g/L	% Initial Polymer %DW[a]	% Polymer Remaining	C=O/DSS[b]
1A	0	2.54	0.61	24.0	100.0	1.80
1B	0	2.11	0.38	18.3	100.0	1.27
1C	0	2.35	0.53	22.7	100.0	1.42
2	3	2.24	0.53	23.7	104.6	1.90
3	19	2.19	0.41	18.5	80.9	1.02
4	23	2.03	0.32	16.0	63.1	0.97
5	43	2.03	0.17	8.5	33.5	0.56
6	50	1.81	0.12	6.6	23.7	0.44
7	74	2.07	0.13	6.4	25.7	0.38
8	90	2.10	0.10	4.7	19.7	0.44

a: based on cell dry weight

b: the ratio of the carbonyl peak at 170ppm to that of the DSS standard at 0 ppm in the ^{13}C NMR spectra

Table 7.2 Harvest time and the results obtained in the intracellular degradation study of PHPV.

Sample Number	Harvest Time, hours	Cell Yield, g/L	Polymer Yield, g/L	% initial Polymer %DW[a]	AR/DSS[b]
1	0	0.068	0.018	26.0	1.79
2	20	0.081	0.037	45.9	1.27
3	46	0.271	0.091	33.5	4.83
4	67	0.276	0.096	34.6	4.32
5	91	0.294	0.102	34.6	3.22
6	115	0.077	0.026	33.9	3.0

a: based on cell dry weight

b: the ratio of the aromatic peak at 170ppm to that of the DSS standard at 0 ppm in the ^{13}C NMR spectra

Table 7.3 Harvest time and the results obtained in the intercellular degradation study of the blend of PHN/PHPV

Sample Number	Harvest Time, hours	Cell Yield, g/L	Polymer Yield, g/L	% Initial Polymer %DW[a]	% Polymer Remaining
1	0	1.48	0.42	28.0	100.0
1B	0	1.64	0.44	27.0	100.0
1C	0	1.47	0.44	27.0	100.0
2	4	1.43	0.34	23.6	87
3	24	1.47	0.18	12.7	47
4	55	1.4	0.20	14.4	53
5	77	1.3	0.13	9.8	36
6	100	0.6	0.10	16	25

a: based on cell dry weight

Table 7.4 The polymer yield and composition of each sample harvested in the intracellular degradation study of the blend of PHN/PHPV

Sample Number	Polymer Yield, g/L	PHPV Yield[a], g/L	% PHPV[b]	AR/DSS[c]	PHN yield[d] (g/L)	% PHN[e]	C=O/DSS[f]
1A	0.42	0.17	40	1.05	0.26	60	1.07
1B	0.44	0.18	40	1.12	0.26	60	0.90
1C	0.44	0.12	35	1.09	0.22	65	0.88
2	0.34	0.13	39	1.23	0.20	61	0.52
3	0.18	0.11	58	1.11	0.08	52	0.51
4	0.20	0.12	62	0.89	0.08	48	0.47
5	0.13	0.09	65	0.70	0.05	35	0.25
6	0.10	0.07	65	Not Run	0.03	35	Not Run

a: determined as the product of the polymer yield and the fraction of PHPV in the polymer, from ^{1}H NMR spectroscopy

b: weight PHPV/total weight of polymer

c: the ratio of the aromatic peak at 128ppm to the DSS peak at 0ppm

d: determined as the product of the polymer yield and the fraction of PHN in the polymer

e: weight PHN/total weight of polymer

f: the ratio of the carbonyl peak at 170ppm to the DSS peak at 0ppm in the ^{13}C NMR spectra

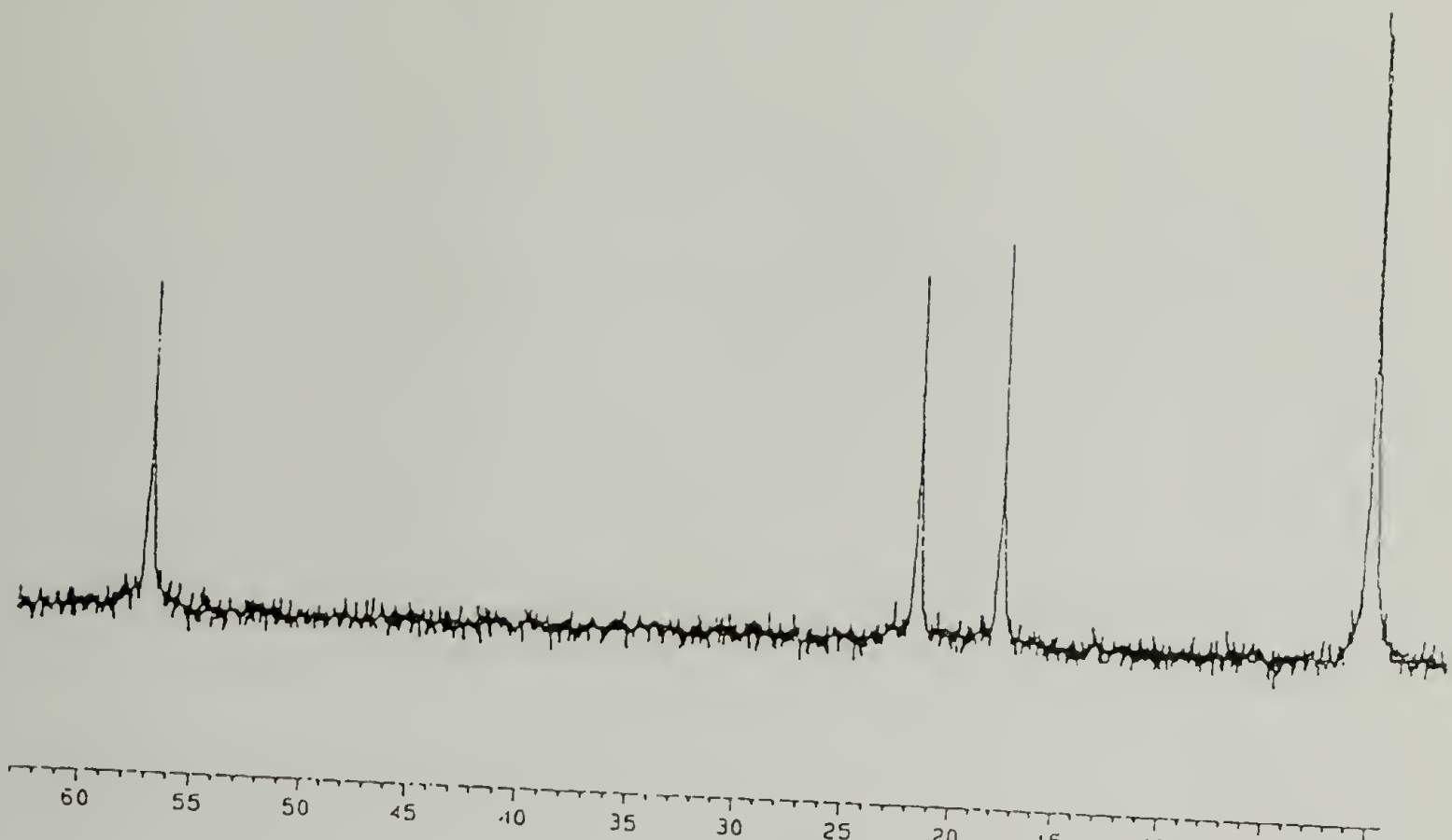

Figure 7.1 ^{13}C NMR spectrum of the internal standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)

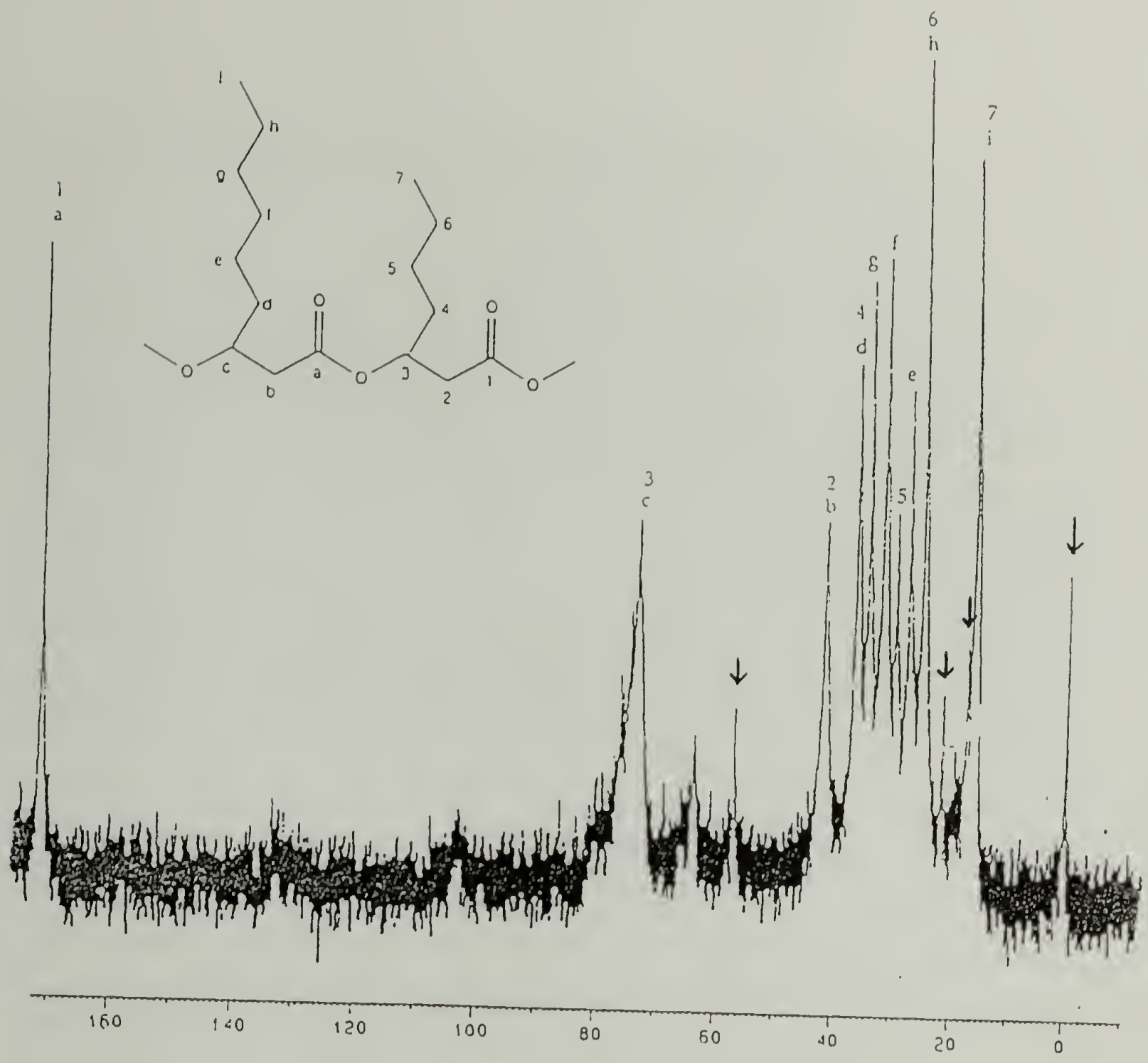

Figure 7.2 ^{13}C NMR spectrum of *P. oleovorans* which contained PHN (peaks for DSS standard are indicated with an arrow).

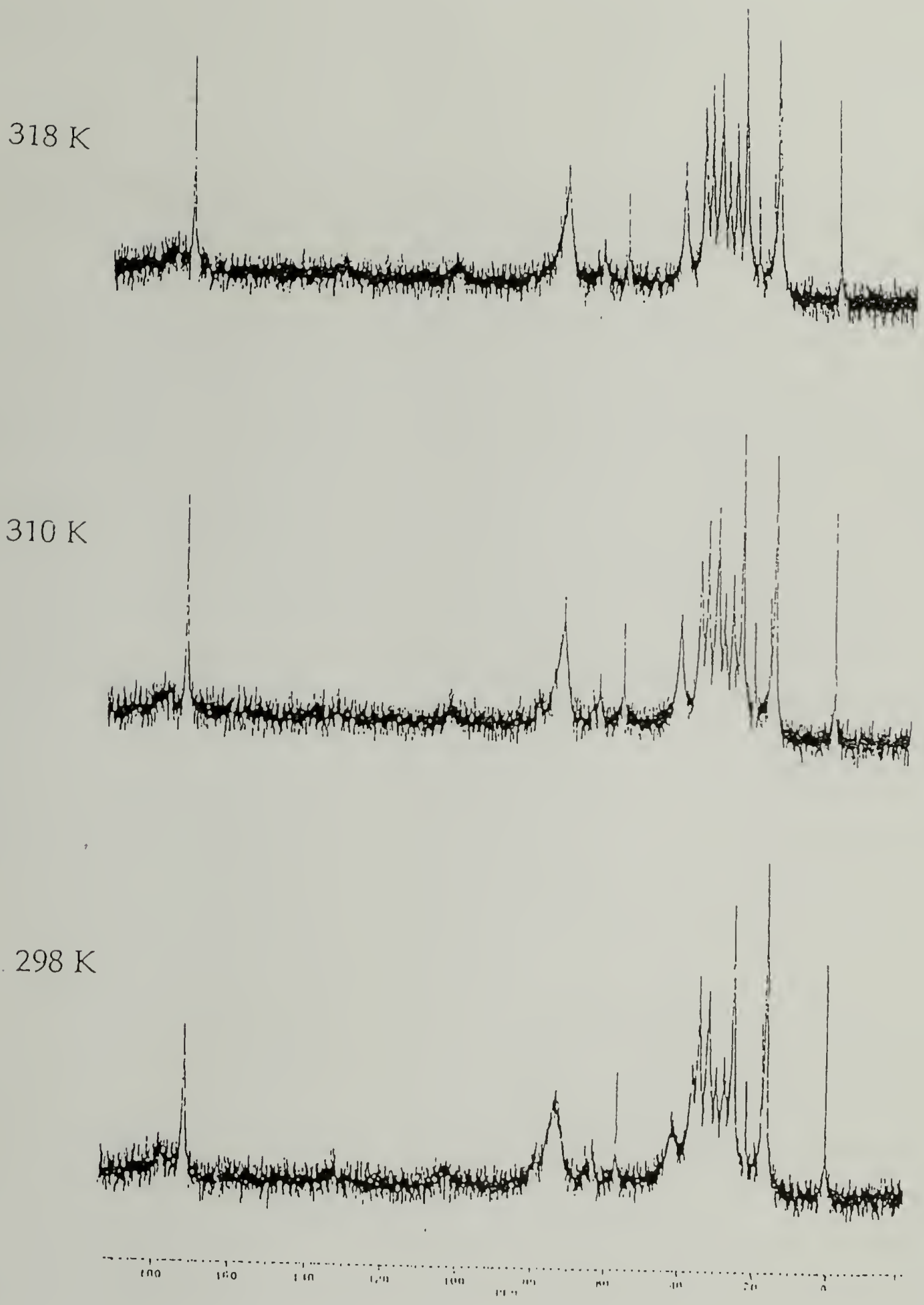

Figure 7.3 ^{13}C NMR spectra of cells containing PHN at three different temperatures, 298, 310, and 318K.

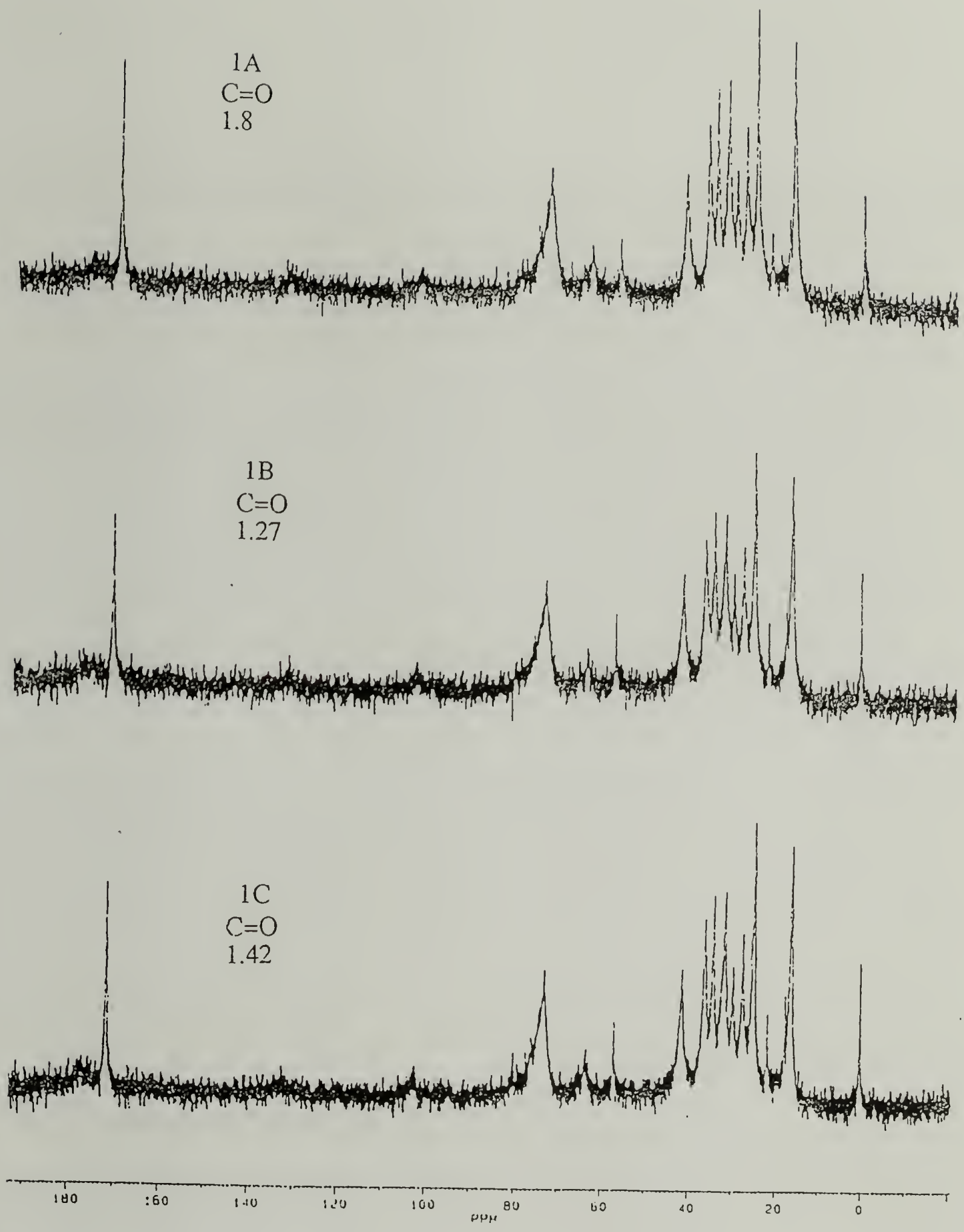

Figure 7.4 ^{13}C NMR spectra of samples 1A, 1B, 1C of *P. oleovorans* which contained PHN as the sole intracellular polymer harvested at time zero

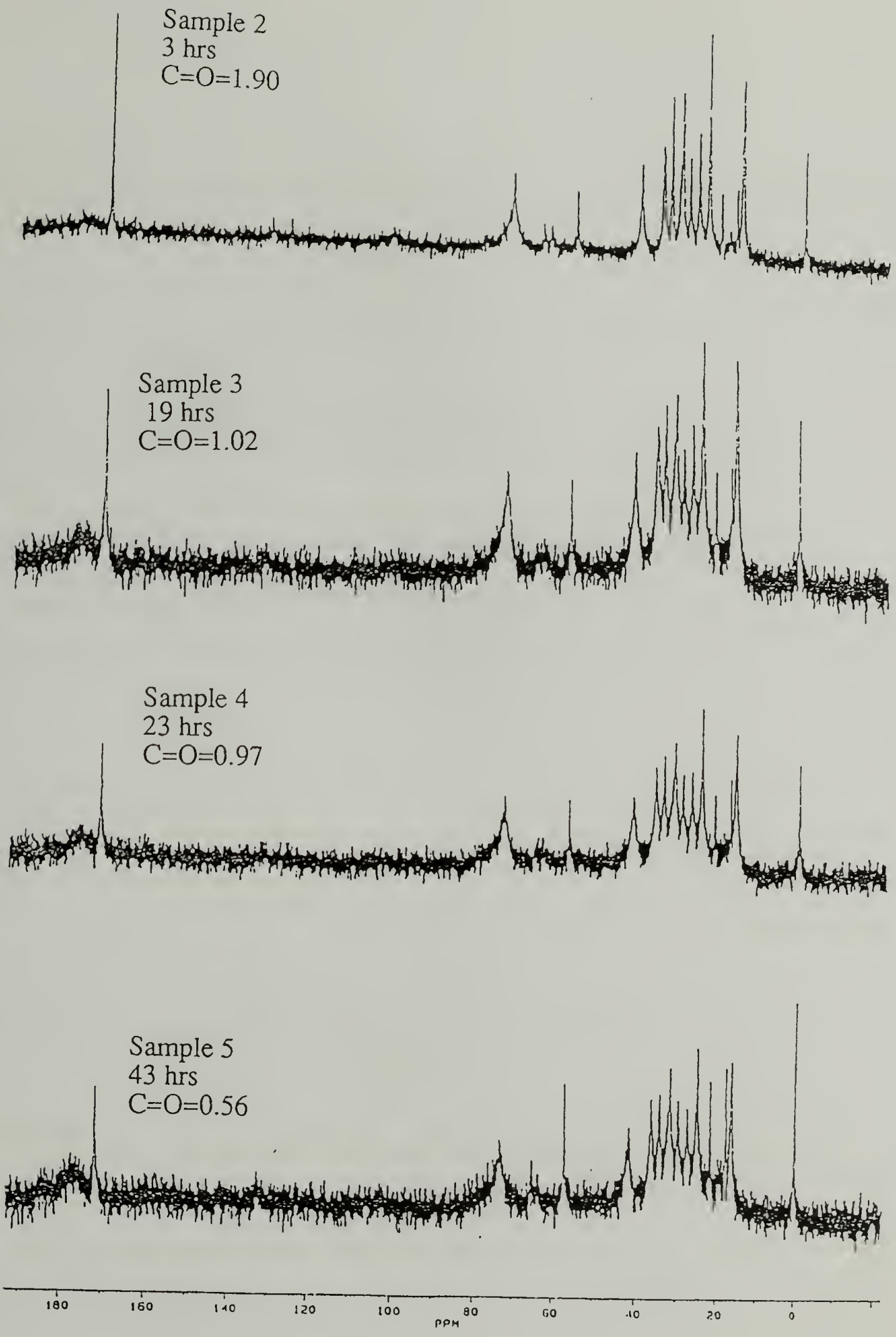

Figure 7.5 ^{13}C NMR spectra of samples 2-8 of *P. oleovorans* which contained PHN as the sole intracellular polymer harvested over a 100 hour period, continued, next page

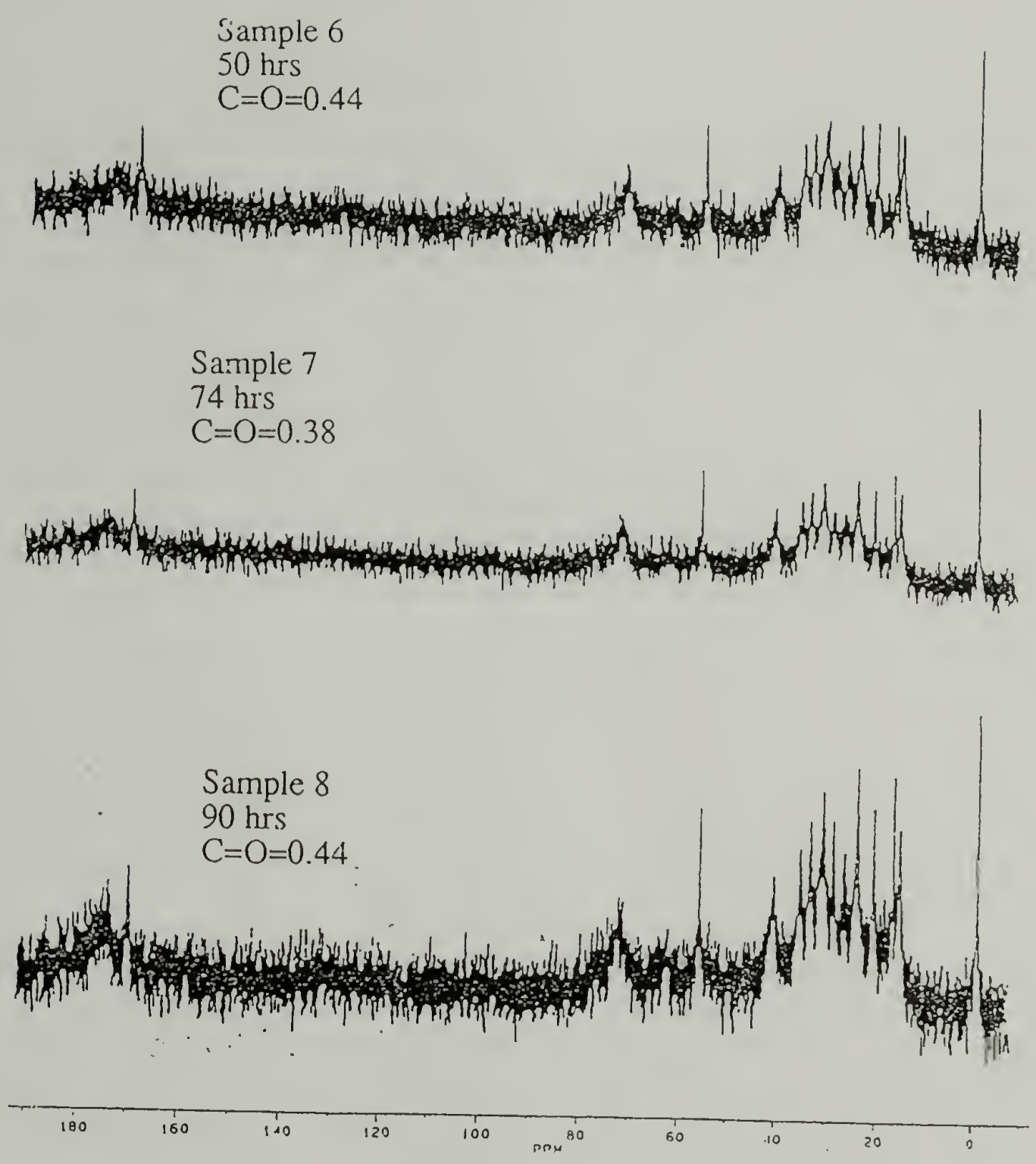

Figure 7.5 continued

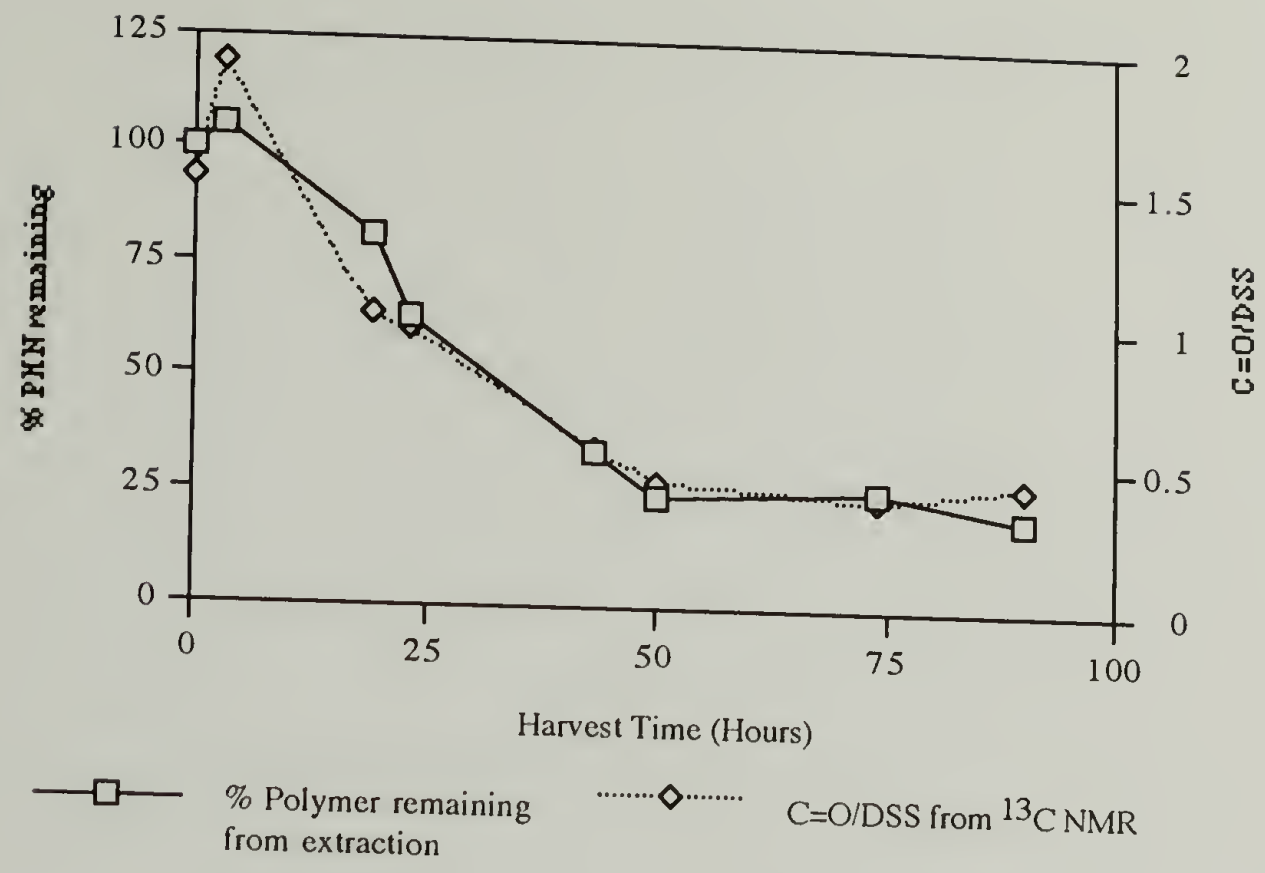

Figure 7.6 PHN remaining (g/L) and C=O/DSS versus harvest time when PHN was the sole intracellular polymer

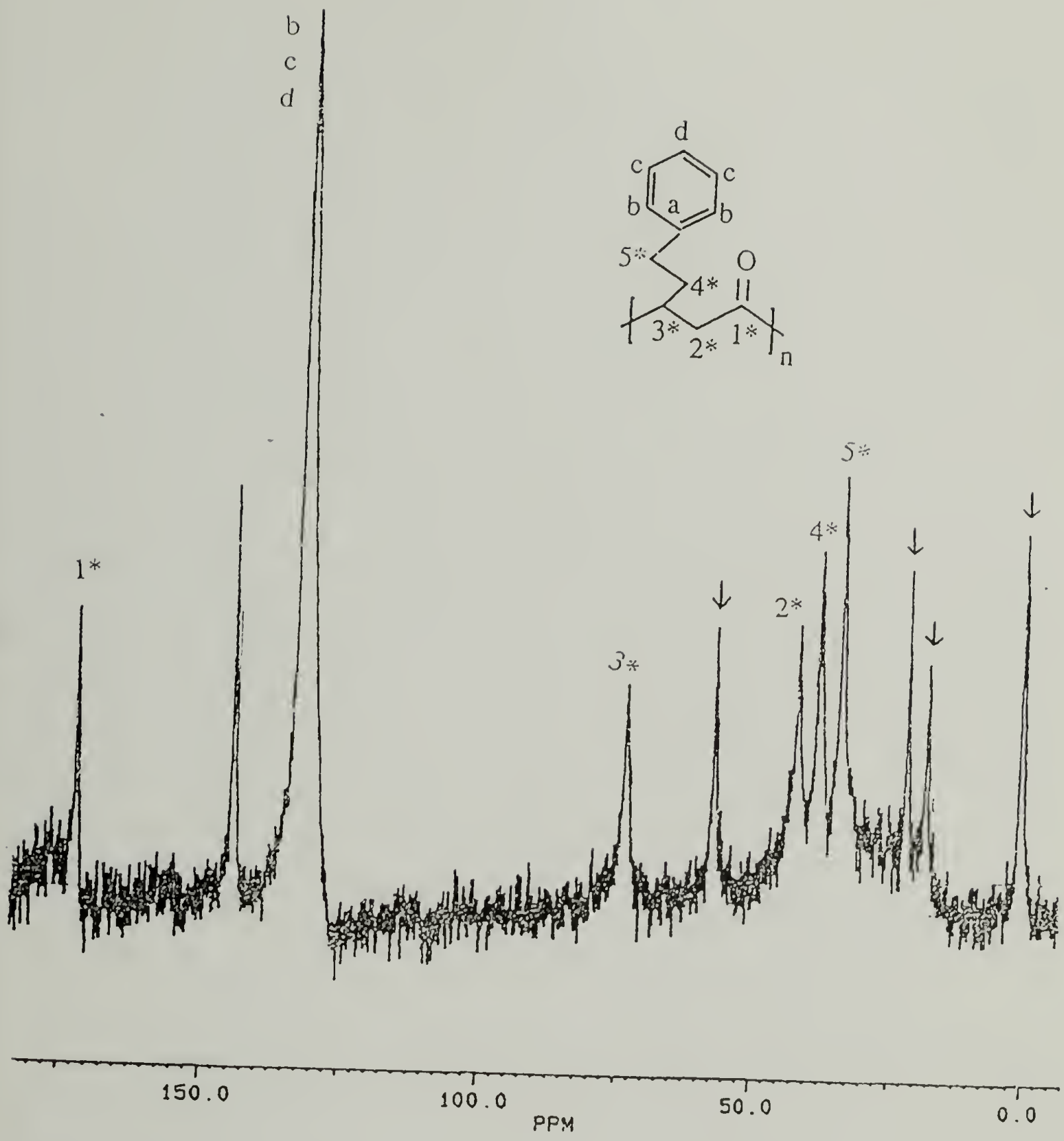

Figure 7.7 ^{13}C NMR spectrum of *P. oleovorans* which contained PHPV (peaks due to the DSS standard are indicated with an arrow).

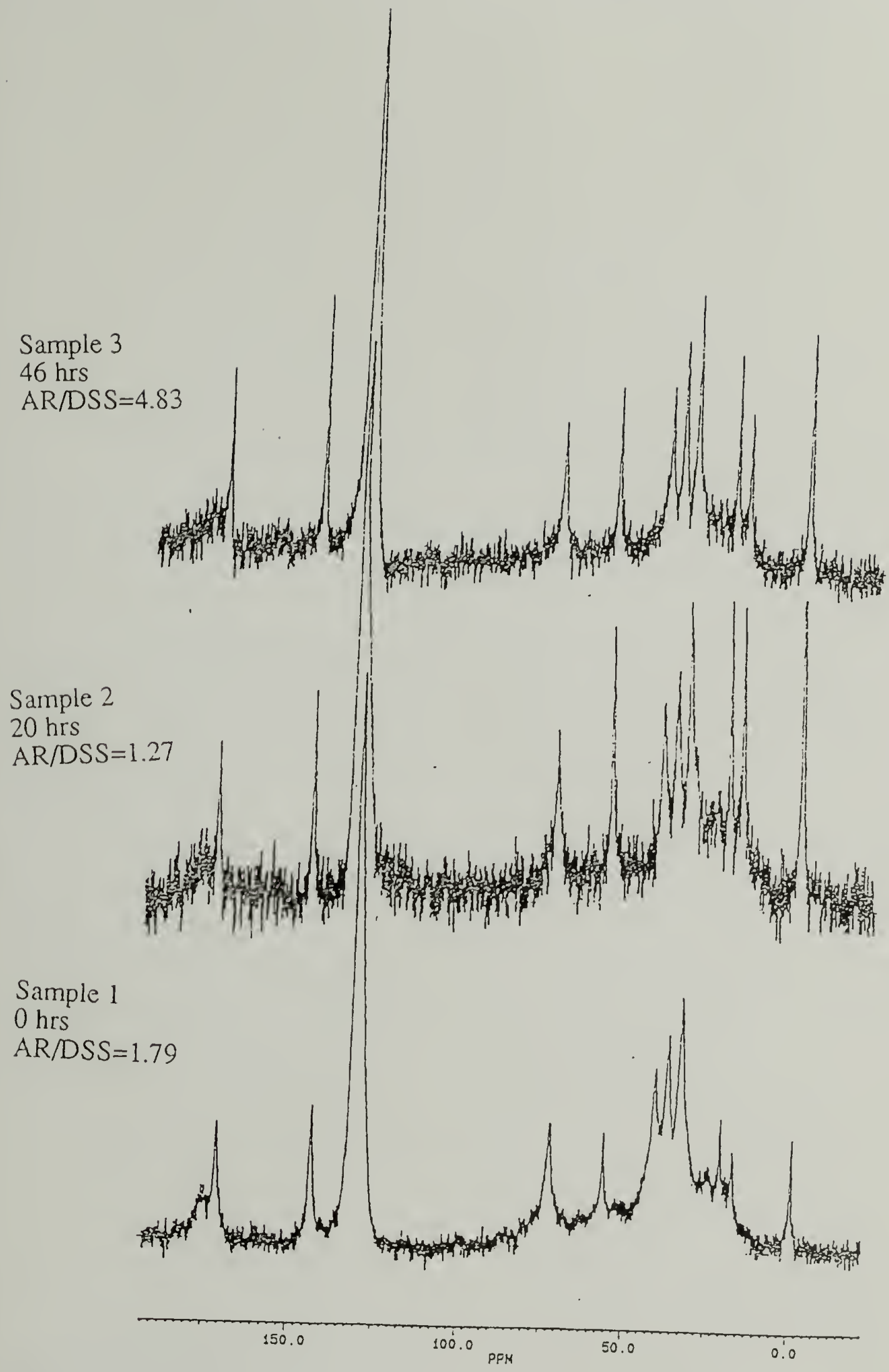

Figure 7.8 ^{13}C NMR spectra of samples 1-6 of *P. oleovorans* which contained PHPV as the sole intracellular polymer, harvested over a 115 hour period

continued next page

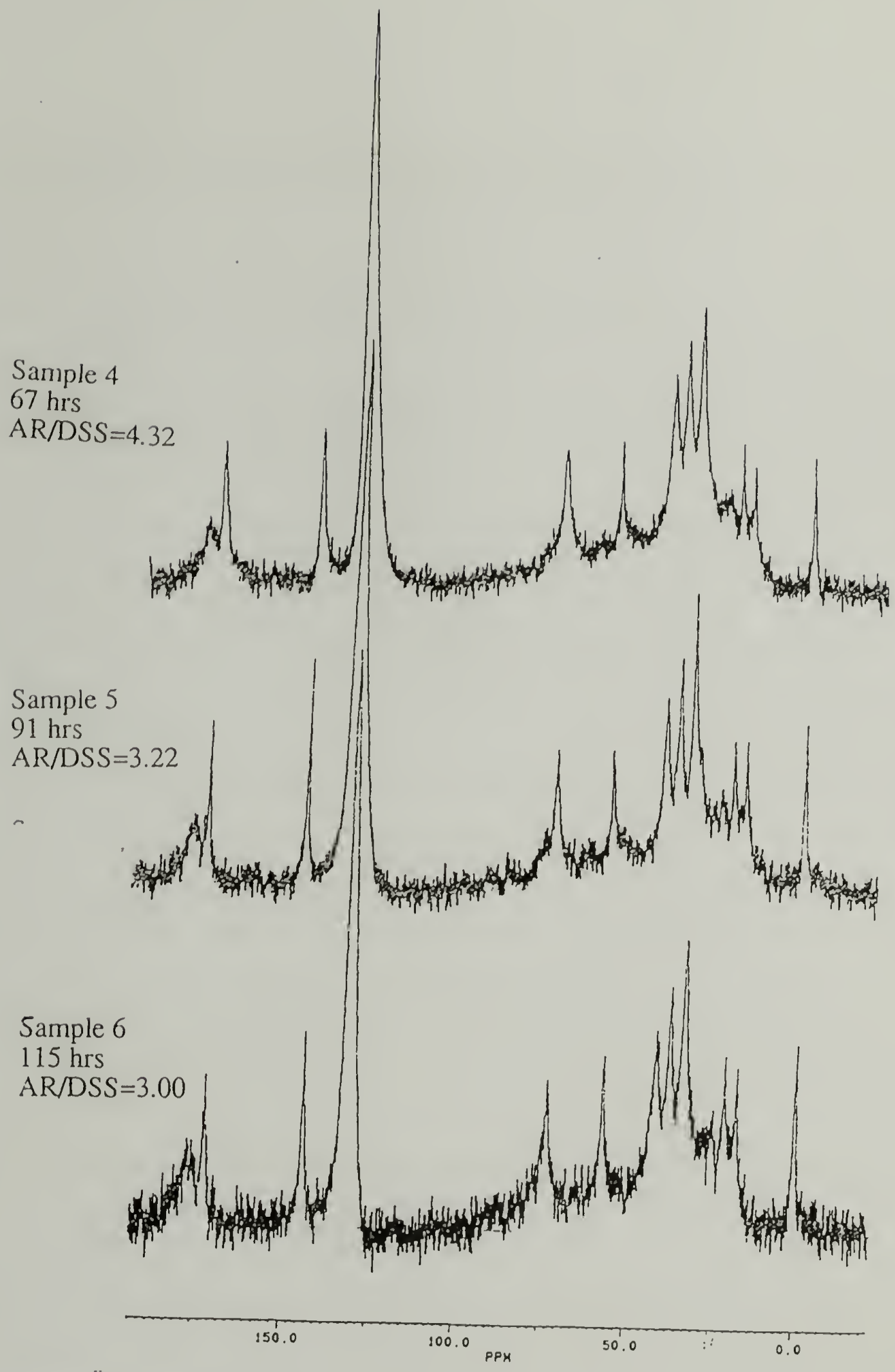

Figure 7.8 continued

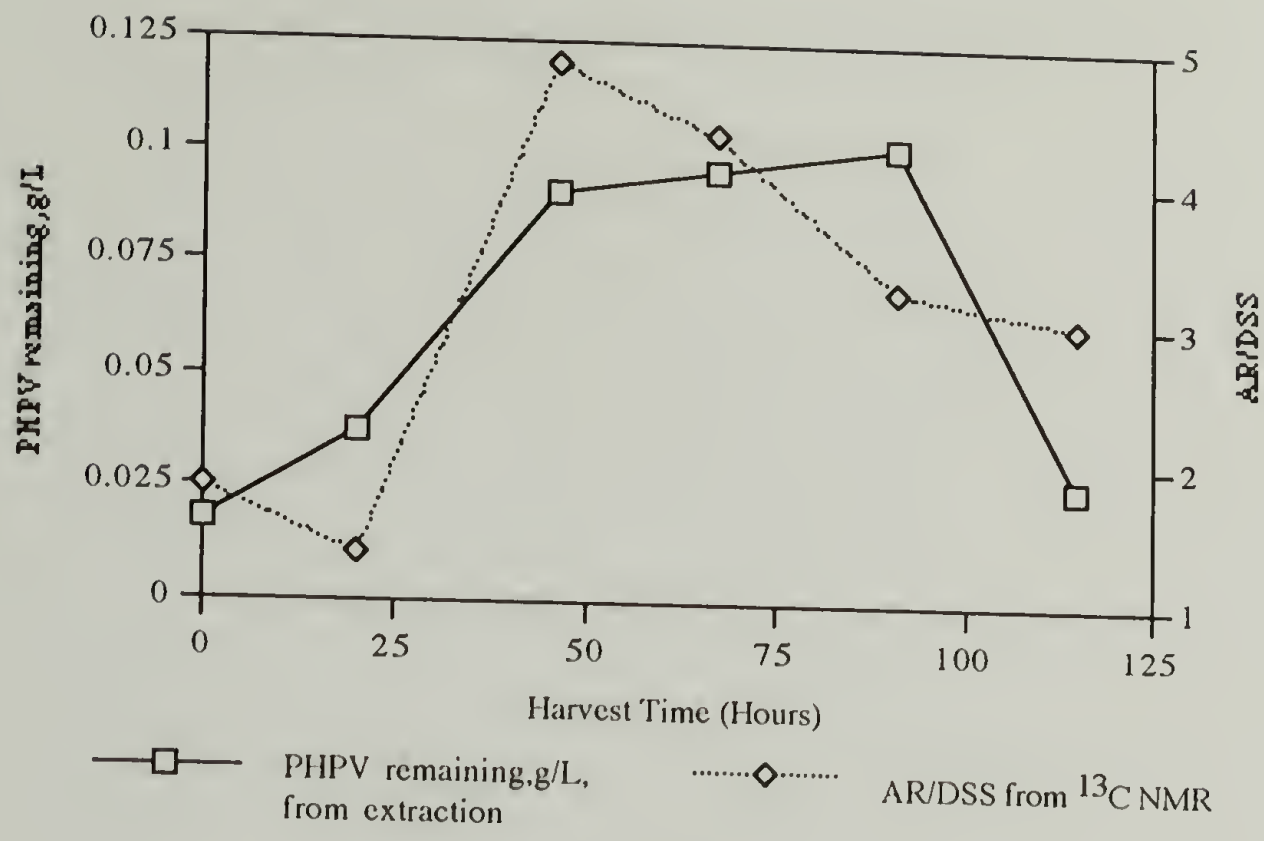

Figure 7.9 PHPV remaining, g/L, and AR/DSS versus harvest time when PHPV was the sole intracellular polymer present .

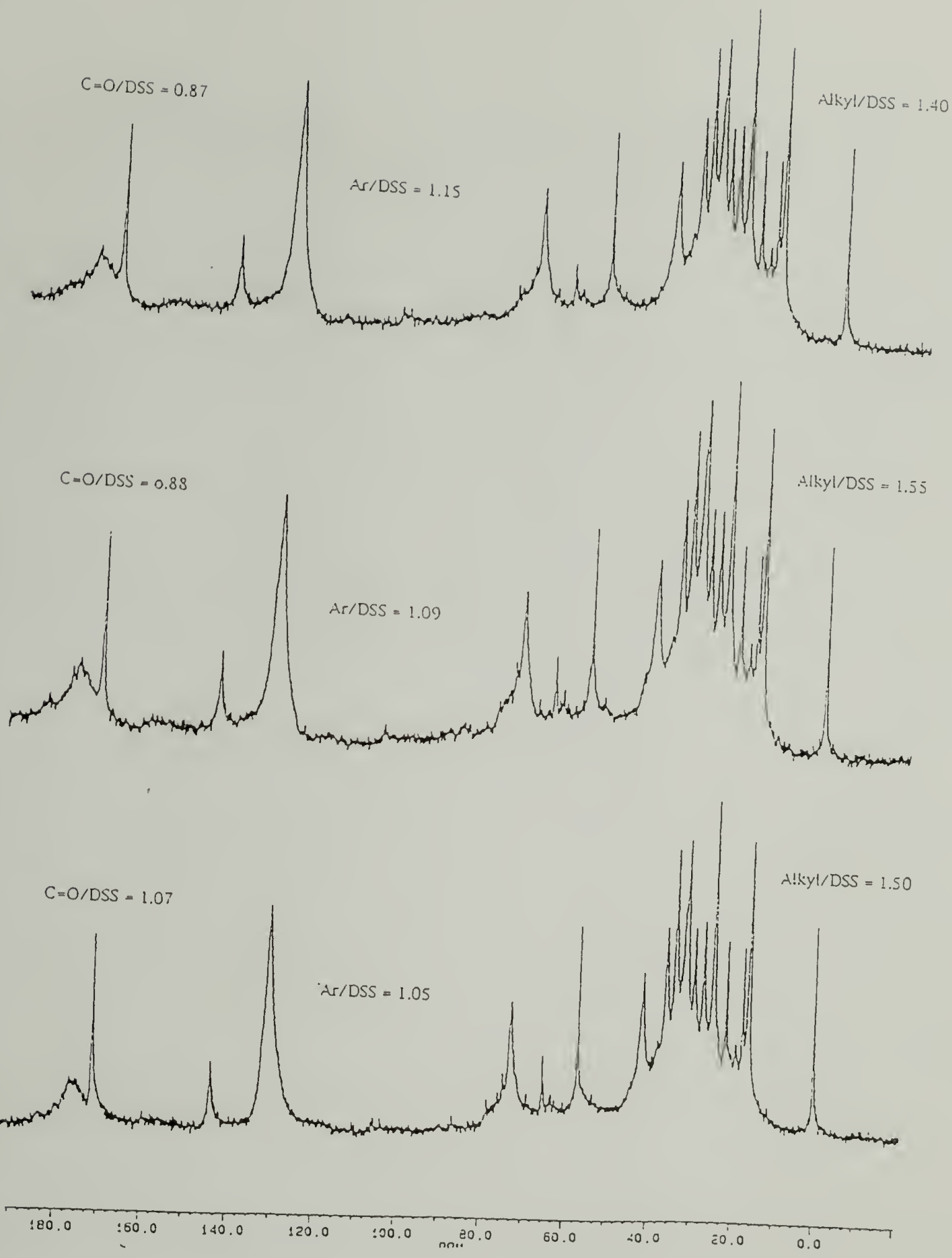

Figure 7.10 ^{13}C NMR spectra of samples 1A, 1B, and 1C of *P. oleovorans* which contained both PHN and PHPV as intracellular polymers

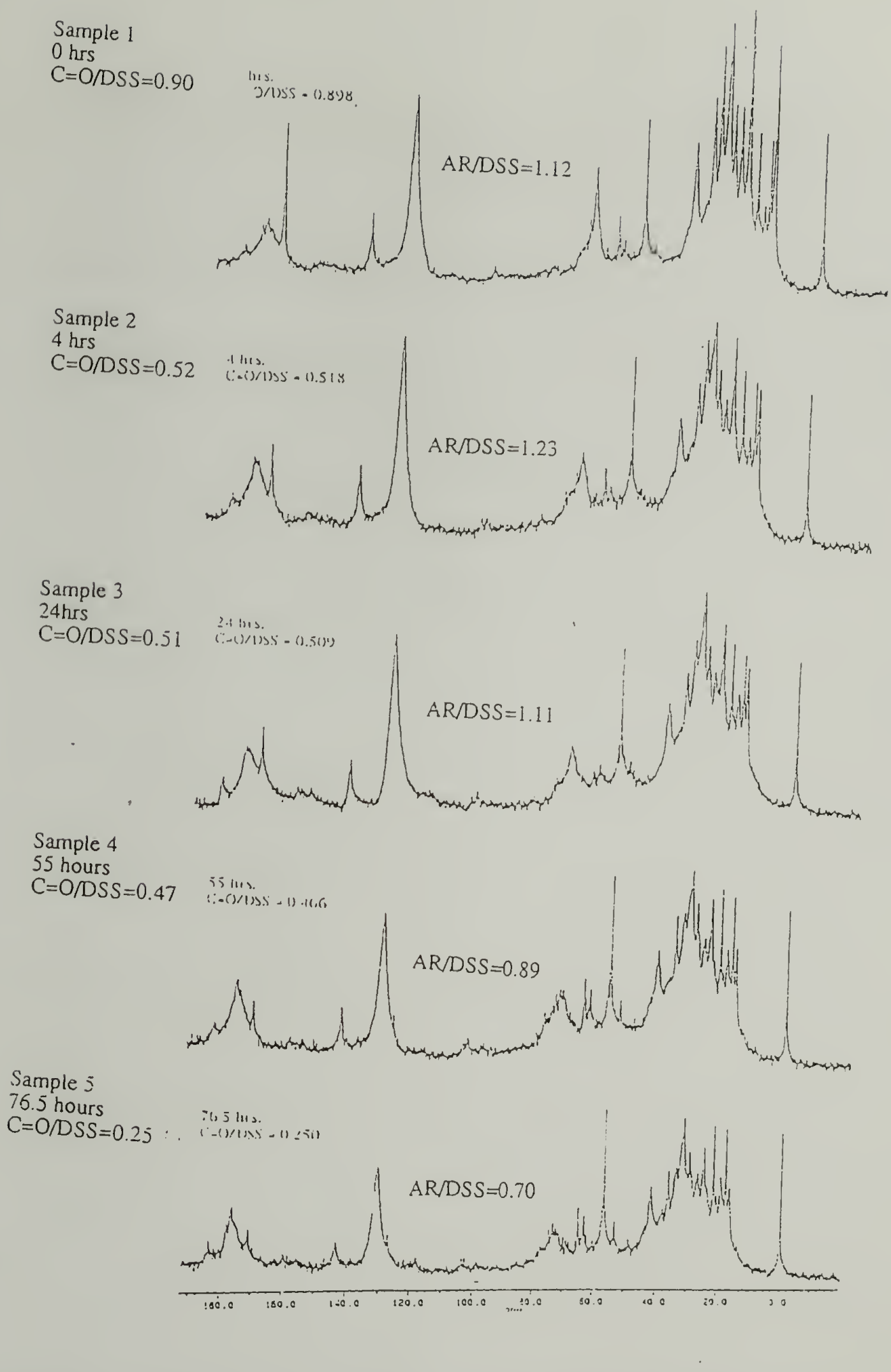

Figure 7.11 ^{13}C NMR spectra of samples 1-5 of *P. oleovorans* which contained both PHN and PHPV harvested over a 100 hour period

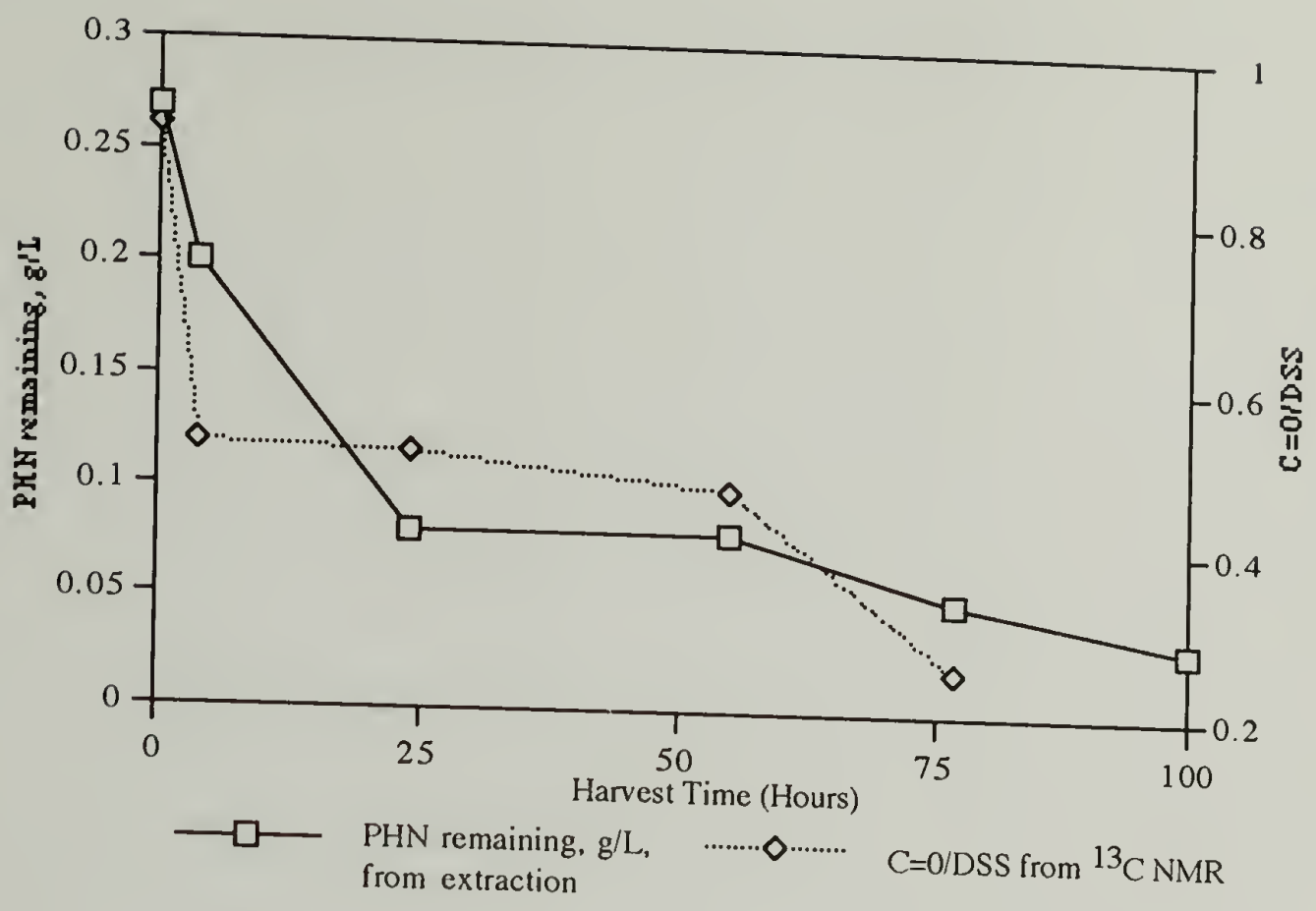

Figure 7.12 % PHN remaining and C=O/DSS versus harvest time when both PHN and PHPV were present as intracellular polymers in *P. oleovorans* .

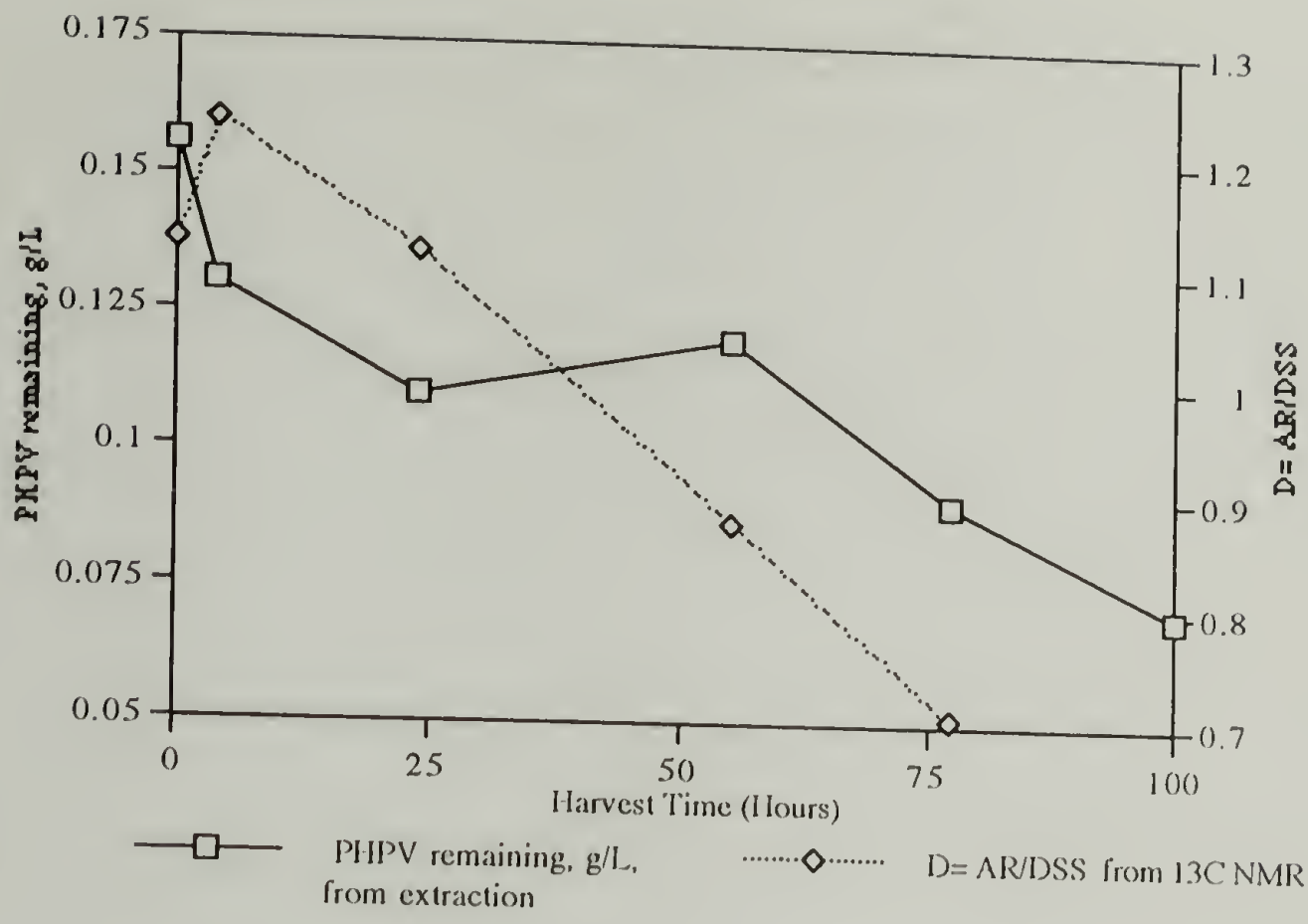

Figure 7.13 % PHPV remaining and D=AR/DSS versus harvest time when both PHN and PHPV were present as intracellular polymers in *P. oleovorans* cells.

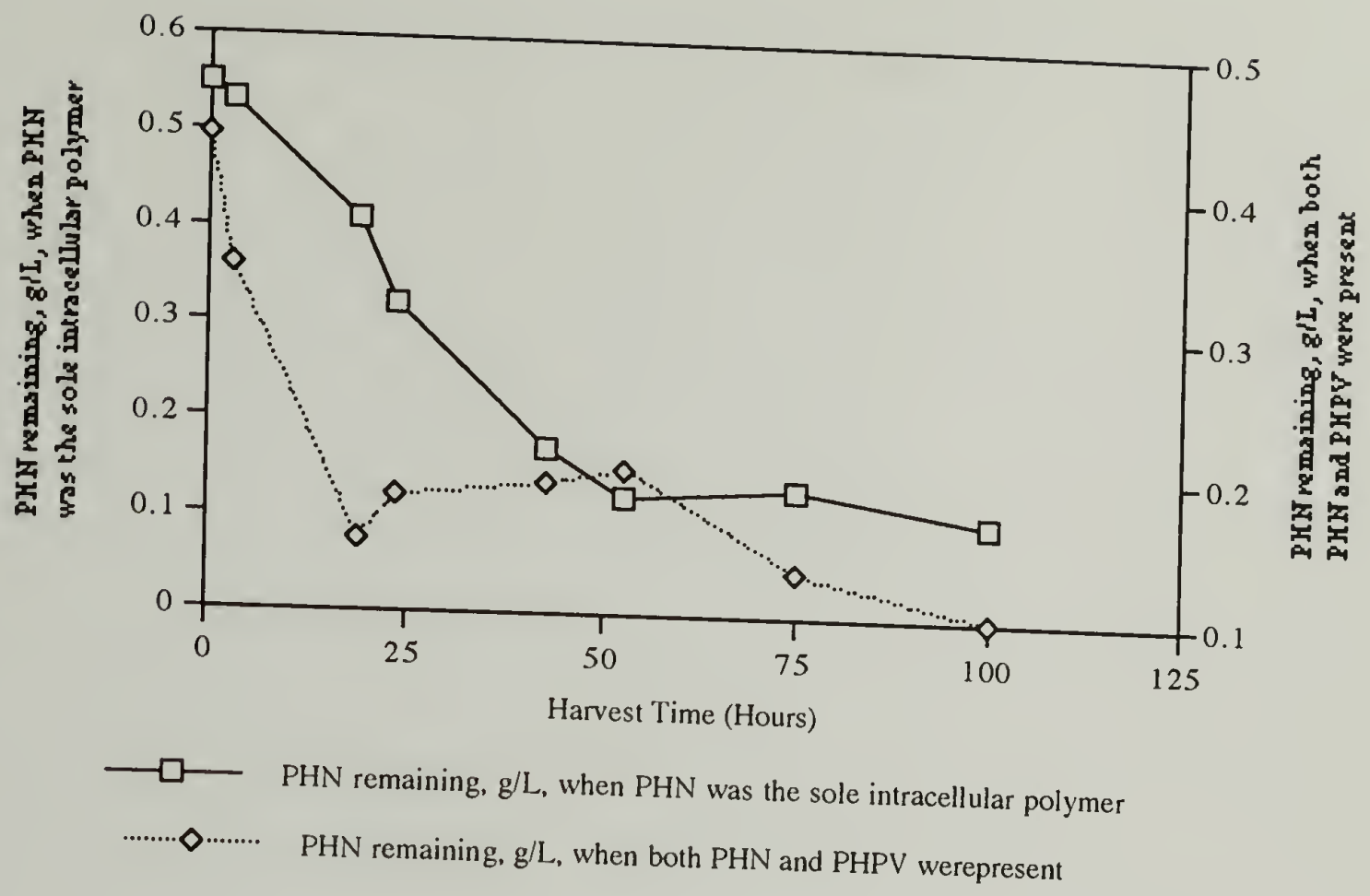

Figure 7.14 Rate of degradation of PHN when it was either the sole intracellular storage polymer, or when PHPV was also present in the granule.

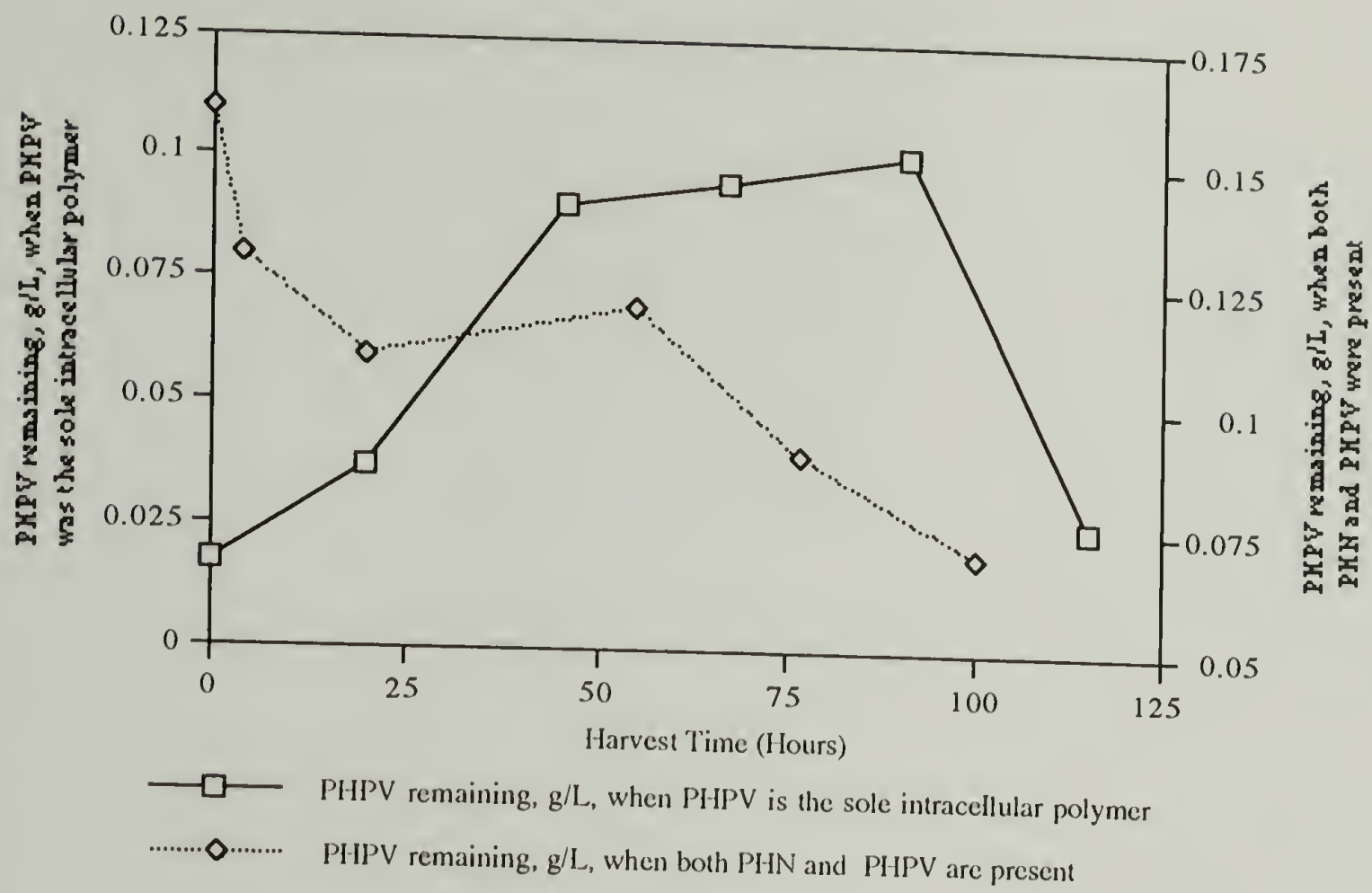

Figure 7.15 Rate of PHPV degradation when it was either the sole intracellular storage polymer and when PHN is also present in the granule.

7.4 Conclusions

In cells which contained PHN as the sole intracellular storage polymer, intracellular degradation was observed 19 hours after the cells were resuspended in a carbon-free medium, and after 90 hours, 80% of the PHN had degraded. In contrast, when PHPV was the sole intracellular storage polymer, degradation was not observed until 115 hours after the cells were resuspended in the carbon-free medium, when the polymer yield fell from 0.102g/L at 90 hours to 0.026 g/L at 115 hours. It appears, therefore, that in addition to producing PHPV at a slow rate, *P. oleovorans* also degraded PHPV slower than it degraded PHN.

When *P. oleovorans* contained a mixture of PHN and PHPV, both polymers degraded intracellularly, but PHPV degraded slower that PHN. However, the rate of degradation of PHPV when it was present as a component of a blend was greater that the degradation observed when PHPV was the sole intracellular polymer. It is possible, therefore, that PHN and PHPV were degraded by the same intracellular depolymerase which operated more efficiently in the presence of PHN.

The rate of degradation of PHN is not significantly affected by the presence of the PHPV. That is, PHN degraded at the same rate when it is the sole polymer present *in vivo* as when it is present as a component of a blend of PHN/PHPV.

The extracellular degradation study on PHN carried out in bench scale composting units resulted in some microbial growth on the polymer surface after one month, and an average of 5% weight loss occurred. In the future, it would be desirable to conduct the experiment for longer than 30 days.

The extracellular depolymerase enzyme excreted by *P. maculicola* degraded PHN but did not degrade PHPV. In addition to an absence of a clear zone, the colony growth was limited when PHPV was used, which may indicate that the PHPV or one of its metabolites may be toxic to the bacteria.

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CHAPTER 8

CONCLUSIONS AND FUTURE WORK

8.1 Conclusions and Future Work

Throughout the course of this dissertation the metabolic flexibility of *P. oleovorans* and *P. putida* was probed in experiments to produce bacterial polyesters containing functional pendant groups. Polymers with phenyl-containing sidechains were of particular interest. Previous research in this group showed that when 5-phenylvaleric acid was fed to *P. oleovorans* a homopolymer, poly-3-hydroxyphenylvalerate, was produced. Also, when the bacteria was grown on an equimolar mixture of NA and PVA, a mixture of a homopolymer (PHPV) and a copolymer (PHN) was produced. One aim of this dissertation was to determine the intracellular location of each of these polymers by selectively staining the PHPV with ruthenium tetraoxide, Examination of the granules by transmission electron microscopy revealed that the two polymers were formed sequentially in the same granule with PHN synthesized in the core of the granule and PHPV formed around this core.

SDS-PAGE results indicate that these two polymers were produced by the same enzyme system. The same polymerase enzyme bands were evident in SDS-PAGE of the enzymes isolated from the bacteria which was grown either on PVA to produce PHPV or on NA to produce PHN. No other bands, which may have been due to new or previously undetected polymerase enzymes, were observed when PVA was used as the sole carbon source. This result indicated that when the bacteria were cofed an equimolar mixture of NA and PVA, both PHN and PHPV were synthesized by the same enzyme system.

It was thought that, since both polymers were produced by the same polymer system, that it might be possible to manipulate the bacteria into producing a random copolymer with repeating units from both substrates, by changing the feed conditions and substrates used. However, this objective was not realized. Characterization of the resulting polymer revealed that it was a mixture of PHN and PHPV. It was concluded, that, while

the same enzyme system was responsible for the production of both polymers, there was some distinction between the two substrates resulting in the production of two separate polymers.

Another aim of this dissertation was to produce a crystalline phenyl-containing bacterial polyester. While annealing of PHPV did not result in the induction of any crystallinity, a crystalline phenyl containing polymer poly-3-hydroxy-5-(4'-tolyl)valerate (PHTV), was obtained when *P. oleovorans* was grown on 5-(4'-tolyl)valeric acid. Growth of *P. oleovorans* on 5-phenylvaleric acid and 5-(4'-tolyl)valeric acid resulted in a significant incorporation of the 5-(4'-tolyl)valeric acid substrate into the polymer.In this case, it was not determined whether a random copolymer or a mixture of two polymers was produced.

Growth of *P. oleovorans* on phenylalkanes, which varied in length from phenylpentane (PPen) to phenylnonane (PNon) resulted in polymer production only in the case where PHep was the carbon substrate used. Surprisingly, the resulting polymer contained 90% poly-3-hydroxyphenylvalerate (PHPV) and 10% poly-3-hydroxyphenylheptanoate (PHPH) units, indicating that the optimum alkyl chain length for polymer production was five carbon atoms. This observation appears to be the first report of a microbially produced phenyl-containing polyester with a 3-hydroxy-7-phenylheptanoate repeating unit.

Growth of *P. oleovorans* on octane prior to the addition of various phenylalkanes, (in order to induce the ω-hydroxylase enzyme system) resulted in polymer production when either PPen and PHep was used. In both cases the predominant phenylalkyl repeating unit was 3-hydroxyphenylvalerate, indicating, once again, that the optimum alkyl chain length when a phenylalkyl substrate was used was 5 carbon atoms.

In addition to producing bacterial polyesters, the degradation, both intracellular and extracellular was investigated. The intracellular degradation of cells which contained PHN, PHPV or an equimolar mixture of PHN/PHPV were monitored using ^{13}C NMR

spectroscopy. The intracellular degradation of PHN occurred at a faster rate than the degradation of PHPV. It appears, therefore, that in addition to producing PHPV at a slow rate, *P. oleovorans* also degraded PHPV slower than it degraded PHN.

When *P. oleovorans* contained a mixture of PHN and PHPV, both polymers exhibited intracellular degradation, but PHPV degraded slower that PHN. However, the rate of degradation of PHPV when it was present as a component of a blend was greater that the degradation observed when PHPV was the sole intracellular polymer. It is possible, therefore, that PHN and PHPV were degraded by the same intracellular depolymerase which operated more efficiently in the presence of PHN. The rate of degradation of PHN is not significantly affected by the presence of the PHPV. That is, PHN degraded at the same rate when it is the sole polymer present *in vivo* as when it is present as a component of a blend of PHN/PHPV.

The extracellular degradation study on PHN carried out in bench scale composting units resulted in some microbial growth on the polymer surface after one month, and an average weight loss of 5% occurred. The surface of the polymer appeared pitted. If further extracellular degradation experiment were carried out using PHN or other MCL-PHA's the duration of the experiment should be increased and also polymer surfaces should be examined using scanning electron microscopy. Also, it would be desirable to conduct the experiment for longer than 30 days.

While *P. oleovorans* was the predominant microorganism used throughout the course of this work, a number of substrates were also fed to *P. putida* BM01, a strain which was isolated from sewage sludge. *P. putida* proved to be superior to *P. oleovorans* in that it produced higher O.D.'s, higher cell yields, and higher polymer yields regardless of the substrate on which it was grown. In future *P. putida* would be the microorganism of choice for use in the production of MCL-PHA's.

8.2 The Future of PHA's

The two major cost factors in the production of PHA's are the cost of the carbon source and the cost of extracting the polymer. If these two factors could be overcome the resulting polymer would be more competitive in the commodity plastic market.

Recent developments in the production of PHA's on cheap abundant feedstocks include the production of MCL-PHA's using glucose or sodium gluconate as a carbon source by three separate research groups [1-3]. The main component of the polymer produced was poly-3-hydroxydecanoate, with poly-3-hydroxyoctanoate and poly-3-hydroxydodecanoate as minor components. Also, a mutant of *Azobacter vinelandii* has produced PHB on a variety of unrefined sugars [4]. Another interesting development has been the report of a PHA-producing *E. coli* which lyses at temperatures of 42°C, which negates the need for solvent or enzymes to remove the polymer from the cellular material.

Genetic engineering may also play a major role in the future production of cheap PHA's. The genes from PHA-producing bacteria have been transfered to other microorganisms, for example the genes from *Alcaligenes eutrophus* have been transfered to *Eshericia coli* [5-7]. *E. coli* was chosen because of the wealth of knowledge which exists on the biochemistry of this microorganism. Up to 90% polymer (based on cellular dry weight) has been obtained using this recombinant *E. coli*.

The production of PHA's in plants is also being investigated as an alternative route to the currently used fermentation route. Biomaterials such as starch and lipids which are plant products are very cheap, the market value of starch is currently $0.10/lb compared to $7/lb for 'Biopol'. There are three enzymes involved in the production of PHB using *A. eutrophus*. 3-ketothiolase catalyses the condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA. Then acetoacetyl-CoA reductase reduces the acetoacyl-CoA to form (R)-3-hydroxybutryl-CoA, which is subsequently polmerized to form PHB by the action of PHB synthase [8]. Only the first enzyme in the pathway, the 3-ketothiolase is endogenously present in plants, so the genes encoding the other two enzymes were

transferred and expressed into *Arabidopsis thaliana* (which is a member of the mustard family) [9,10]. By staining sections of the plant and characterizing the polymer obtained using GC-MS it was determined that between 20-100μg PHB/g of plant material was obtained. A negative side-effect of this PHB production was the the fact that the plants growth was stunted. This is due to the diversion of a large amount of precursor away from the normal pathway. Acetoacetyl-CoA is normally converted to mevalonate which is used in the synthesis of a variety of essential components including phytohormones, sterols and cartenoids.

The solution to this side effect may involve the diversion of the carbon away from the production of nonessential storage granules such as starch or lipids to PHB production. Non-essential means that these components are not required for growth or seed production. By this method PHB could be produced without affecting cell growth or seed production. If PHB could be produced in reasonably high quantities in plants, the price of the polymer could be sufficiently reduced so that it could compete in the commodity market.

8.3 References

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